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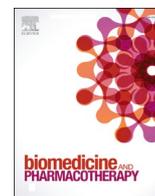
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Mesalazine initiates an anti-oncogenic β -catenin / MUCDHL negative feed-back loop in colon cancer cells by cell-specific mechanisms

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ABSTRACT

Chronic inflammation associated with intestinal architecture and barrier disruption puts patients with inflammatory bowel disease (IBD) at increased risk of developing colorectal cancer (CRC). Widely used to reduce flares of intestinal inflammation, 5-aminosalicylic acid derivatives (5-ASAs) such as mesalazine appear to also exert more direct mucosal healing and chemopreventive activities against CRC. The mechanisms underlying these activities are poorly understood and may involve the up-regulation of the cadherin-related gene *MUCDHL* (*CDHR5*). This atypical cadherin is emerging as a new actor of intestinal homeostasis and opposes colon tumorigenesis. Here, we showed that mesalazine increase mRNA levels of *MUCDHL* and of other genes involved in the intestinal barrier function in most intestinal cell lines. In addition, using gain / loss of function experiments (agonists, plasmid or siRNAs transfections), luciferase reporter genes and chromatin immunoprecipitation, we thoroughly investigated the molecular mechanisms triggered by mesalazine that lead to the up-regulation of *MUCDHL* expression. We found that basal transcription of *MUCDHL* in different CRC cell lines is regulated positively by CDX2 and negatively by β -catenin through a negative feed-back loop. However, mesalazine-stimulation of *MUCDHL* transcription is controlled by cell-specific mechanisms, involving either enhanced activation of CDX2 and PPAR- γ or repression of the β -catenin inhibitory effect. This work highlights the importance of the cellular and molecular context in the activity of mesalazine and suggests that its efficacy against CRC depends on the genetic alterations of transformed cells.

1. Introduction

Inflammatory Bowel Diseases [IBD: Crohn's Disease (CD) and Ulcerative Colitis (UC)] are chronic diseases with a growing incidence that already affect over 6 million people worldwide [1]. They are usually characterized by alternating remission and recurrence periods where patients suffer from abdominal pain, diarrhea and/or bleeding [2]. As a result, these diseases can lead to structural bowel damage, loss of function and increased need for surgery. Moreover, chronic inflammation in the colon is known to contribute to carcinogenesis following epithelial dysplasia and several studies have shown that the risk to

develop colorectal cancer (CRC) is 2–5 times higher in those patients than in the general population [3,4]. The molecular pathogenesis of colitis-associated cancer (CAC) involves the same mechanisms of genetic instability occurring in sporadic CRC (sCRC), but the frequency and timing of specific mutation may be different (e.g. *TP53* mutations much more common in CAC than sCRC and the opposite for *APC*) [5].

Currently, available IBD treatments [i.e. 5-aminosalicylic acid derivatives (5-ASAs), corticosteroids, thiopurines, methotrexate, biological therapies, or Janus Kinase (JAK)-1 inhibitors] aim to extend remission periods but none of them are curative and patients with IBD generally require long-term maintenance therapy [6]. For mild to

Abbreviations: Ab, Antibody; 5-ASAs, 5-aminosalicylic acid derivatives; Cdhr, Cadherin-related; CRC, colorectal cancer; HRP, Horseradish peroxidase; IBD, Inflammatory Bowel Diseases; IMAC, Intermicrovillar Adhesion Complex; UC, Ulcerative Colitis.

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moderate UC, especially those with left-sided or extensive disease, 5-ASAs represent the first line treatment and effectively reduce intestinal inflammation [7,8]. Interestingly, 5-ASAs (especially mesalazine) were also shown to improve mucosal healing and decrease the risk of CRC [9]. Conflicting data have been obtained about the chemopreventive activity of 5-ASAs but several meta-analyses concluded that mesalazine halves the risk of developing CAC or dysplasia in UC patients [9]. The mechanisms underlying this specific activity remain incompletely understood as 5-ASAs are not only able to lower inflammation, but also reduce proliferation, induce apoptosis and improve replication fidelity in colon cancer cell lines [10–12]. Interestingly, a very recent study has also shown that 5-ASAs lead to membrane relocation of junction proteins such as E-cadherin, thereby restoring the intestinal barrier [13,14]. However, expression profiling of various cadherins in 5-ASAs-treated colon cancer cells indicated that the atypical cadherin MUCDHL showed the strongest stimulation [15].

MUCDHL (also called CDHR5) belongs to the heterogeneous and poorly-studied cadherin-related (CDHR) family that regroups the most divergent members of the cadherin superfamily [16,17]. MUCDHL is highly expressed in the intestinal epithelium and, while it is localized at intercellular junctions like classical cadherins, its main location is at the apical membrane of absorptive cells where it was shown to link microvilli of the brush border together through the intermicrovillar adhesion complex (IMAC) [18–20]. Several studies reported a reduced MUCDHL expression in both CRC and IBD [21–23]. Consistently, we showed that diminished MUCDHL expression exacerbates tumorigenesis both in mouse and human, while enforced expression in CRC cells opposes tumor growth in nude mice [24,25]. In light of the implication of MUCDHL in CRC and possibly in IBD, its up-regulation by mesalazine is interesting as it suggests that this gene might mediate some of its mucosal healing and/or chemopreventive activity. This hypothesis is reinforced by the observation that both mesalazine and MUCDHL inhibit the transcriptional activity of β -catenin, which plays a prominent role in intestinal cell proliferation, tissue repair and tumorigenesis [26]. Indeed, the up-regulation of MUCDHL by 5-ASAs results in enhanced localization of β -catenin at adherens junctions at the expense of its nuclear localization [15,23].

Here, we investigated the molecular mechanisms by which mesalazine stimulates MUCDHL expression. Our data emphasized the importance of the molecular and cellular context for the activity of 5-ASAs, as we identified several transcription factors that cell-specifically relay its impact on MUCDHL expression.

2. Materials and methods

2.1. Cell lines and reagents

Human colon adenocarcinoma DLD-1, SW480, Caco-2, HCT116 and HT29 cells (American Type Culture Collection) were maintained at 37 °C and grown in RPMI-1640 (Gibco) for DLD-1 and Dulbecco's modified Eagle medium (D. Dutscher) for the other cell lines. Culture media were supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (Gibco). All experiments were performed with cells passaged less than ten times after being thawed.

Troglitazone was purchased from Bertin Pharma and solubilized in DMSO. Mesalazine (here called 5-ASA) was from Ferring Laboratory and was dissolved in culture medium at concentrations that ranged from 10 mM to 40 mM at pH 7.4, corresponding to the tissue concentrations found in the colon of patients treated with 2, 3 or 4 g/d of 5-ASA. Control cells were treated with an equivalent amount of vehicle.

2.2. Plasmids

pGL3-basic and pRL-null were purchased from Promega, pcDNA/TO from Invitrogen, pFLAG-CMV2 from Sigma and pBSK(-) from Stratagene. pMUCDHL 1.4 kb-Luc in pGL3-basic vector was created in our

laboratory [25] and pTopFlash (i.e. luciferase reporter gene controlled by 3 TCF binding sites, [27]) was obtained from H. Clevers (Utrecht University Medical Center, Utrecht, The Netherlands).

cDNAs encoding human PPAR- γ were generated by reverse transcription using SuperScript III reverse transcriptase (Invitrogen), a specific primer (CCTCAGAATAGTGCAACTGGAA) and 2 μ g of RNA extracted from HT29 cells. Using these cDNAs, a 1449 bp fragment was first amplified by PCR (Platinum® Pfx Polymerase, Invitrogen) with a forward (CGATGACGACAAGCTTCTGACCATGGTTGACACAG) and a reverse primer (CCCGGGATCTCTAGACTAGTACAAGTCCTGTAGA), then sub-cloned into HindIII/XbaI-linearized pFLAG-CMV2 using the In-Fusion® HD Cloning kit (Clontech Laboratories, Inc). The coding sequence of the resulting pFLAG-hPPAR- γ 1 plasmid was verified by sequencing (GATC).

2.3. Small interfering RNA

Silencer Select small interfering RNA (siRNA) were from Ambion and included: siCt (Negative control #2), siPPAR- γ #1 (s10888), siPPAR- γ #2 (s10886), siCDX2#1 (s2877), siCDX2#2 (s2878), si β -catenin#1 (s437), si β -catenin#2 (s438), siTCF4#1 (s13881), siTCF4#2 (s13880).

2.4. Transfections

For overexpression experiments (500,000 cells/well in 6-well plates, 1 μ g DNA) and chromatin immunoprecipitations (1.10⁶ cells/well in 3004 plates, 2.4 μ g DNA), cells were transfected with plasmidic DNA using Jetprime (Polyplus Transfection) and treated or not 6 h later with 30 mM of 5-ASA for 48 h. For Luciferase assays (150,000 cells/well in 24-well plates, 600 ng DNA), cells were transfected with various combinations of pGL3-basic, pMUCDHL 1.4 kb-Luc, pBSK(-) (promoter assays) and pRL-null or of pcDNA/TO, pTopFlash and pRL-null (TopFlash assays) using JetPEI (Polyplus Transfection) and treated or not 24 h later with 10–30 mM of 5-ASA for 24 h.

For siRNA experiments, 500,000 cells/well in 6-well plates were transfected with siRNA at a final concentration of 10 nM per well using Lipofectamine RNAiMAX (Invitrogen). After 6 h of transfection, cells were treated with 5-ASA 30 mM for 48 h.

2.5. RNA extraction and RT-qPCR

Total RNA was isolated using TRI Reagent (MRC). Reverse transcription (RT) was performed with 2 μ g of RNA in 20 μ L using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). 4 μ L of diluted RT (5X) were mixed with FastStart TaqMan Buffer (Roche) and gene specific TaqMan probes (TaqMan Gene Expression Assays, Applied Biosystems) to perform quantitative PCR using 7500 real-time PCR System (Applied Biosystems). Analysis of the results was performed with the 7500 software v2.0.5 (Applied Biosystems) using the relative $\Delta\Delta$ Ct quantification method and the expression of the housekeeping gene TBP for normalization [28]. The following TaqMan Gene Expression Assays were used: CDHR5 M and L isoforms (MUCDHL) Hs01059246_m1; CDX2 Hs00230919_m1; PPAR- γ Hs01115513_m1; CTNBN1 (β -catenin) Hs00355049_m1; CDHR2 Hs00214362_m1; USH1C (Harmonin) Hs01559312_m1; MYO7B Hs00826900_m1; TCF7L2 (TCF4) Hs01009044_m1; TBP Hs00427620_m1.

2.6. Chromatin immunoprecipitation (ChIP)

Fixation of cells was performed with 1% (vol/vol) formaldehyde for 10 min at room temperature and quenched with 0.125 mol/L glycine for 5 min. ChIP experiments were carried out using the EZ-Magna ChIP G Chromatin Immunoprecipitation kit as recommended by the supplier (Millipore) [29]. Sonicated cross-linked chromatin from 1.10⁶ cells was incubated over night at 4 °C with 4 μ g of antibody: mouse immunoglobulin G (IgG) (Upstate Cell Signaling); mouse anti-FLAG (clone M2,

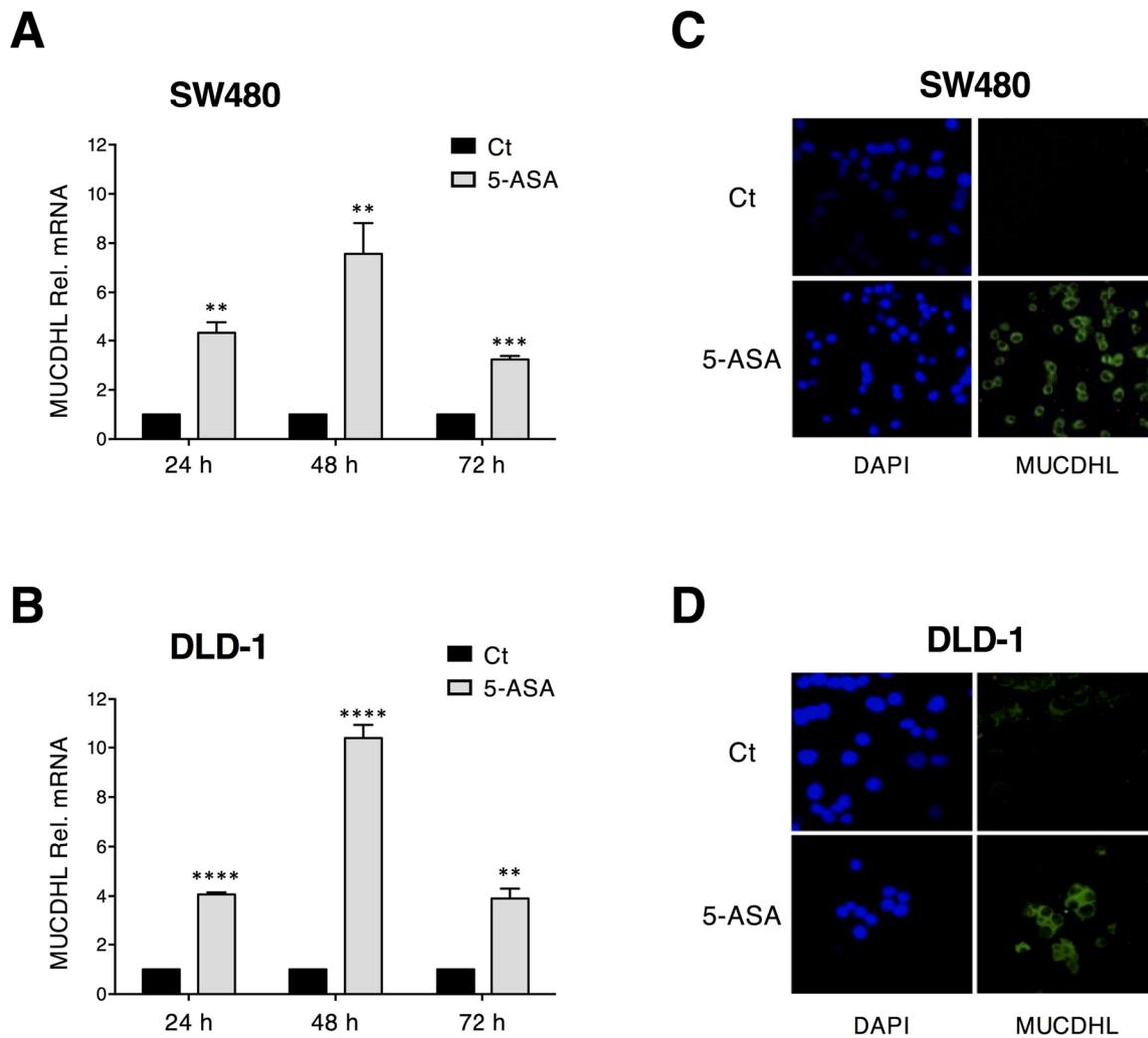


Fig. 1. Impact of 5-ASA on *MUCDHL* expression in CRC cell lines. (A) RT-qPCR analyses of SW480 cells untreated (Ct) or treated for the indicated time with 30 mM of 5-ASA. *MUCDHL* mRNA levels (normalized with those of a housekeeping gene) in untreated cells were set at 1 for each time point and data correspond to average $RQ \pm SEM$ ($n = 3$, untreated and treated cells were statistically compared for each time point using a Student *t*-test). (B) as (A) for DLD-1 cells. (C) Immunofluorescence detection of *MUCDHL* protein expression (green) and DAPI nuclear staining (blue) in SW480 cells treated or not with 30 mM of 5-ASA for 48 h. Original magnification 100x. (D) as in (C) for DLD-1 cells.

Sigma); mouse anti- β -catenin (clone 14, BD Transduction lab); mouse anti-TCF4 (D-4, Santa Cruz Biotechnology) and mouse anti-CDX2 (clone 88, BioGenex). Enrichment was measured by qPCR using FS Universal SYBR Green MasterRox mix (Roche) with 7500 real-time PCR System (Applied Biosystems). Primers used for PPAR- γ , CDX2, β -catenin or TCF4 located in the human *MUCDHL* promoter and positive controls are listed in Fig. A1.

2.7. Luciferase assays

Luciferase assays were performed with the Dual-Luciferase Reporter Assay (Promega) as recommended by the supplier. Luciferase activities of three technical replicates were measured using TriStar² LB942 from Berthold.

2.8. Sub-cellular fractionation and Western blot

Cells were seeded in 6-well plates for 24 h and treated with 5-ASA 30 mM for 6 h. Sub-cellular fractionation was performed with NE-PER kit (Pierce) as recommended by the supplier using 2.10^6 cells (10 μ L packed cell volume). Protein concentrations were measured using Bio-Rad Protein Assay. 20 μ g of proteins were separated by SDS-PAGE 15%

and transferred to 0.2 μ m Hybond PVDF membranes (Amersham). Membranes were saturated at room temperature for 45 min in PBS-Tween 0.1% containing 1% BSA or 5% non-fat dry milk. Membranes were incubated at 4 $^{\circ}$ C overnight with one of the following primary antibodies: mouse monoclonal PPAR- γ (E-8, 1/1000; Santa Cruz Biotechnology), goat polyclonal GAPDH (V-18, 1/10000; Santa Cruz Biotechnology) and rabbit monoclonal Histone H3 (clone D1H2 XP, 1/2000; Cell Signaling Technology). The following secondary antibodies were incubated for 45 min at room temperature: anti-mouse IgG HRP-linked (1/5000; Cell Signaling Technology), anti-goat IgG HRP-linked (1/10000; Santa Cruz Biotechnology) or anti-rabbit IgG HRP-linked whole Ab (1/10000, Amersham). Signals were detected using ECL (Amersham).

2.9. Immunofluorescence

Immunofluorescence was performed on fixed cells (4% PFA, 20 min) grown on glass Lab-Tek chamber slides system 8 wells (Nunc). After permeabilization (PBS-Triton 0.1%, 10 min), cells were blocked with Normal Goat Serum 10% for 1 h (Sigma) and incubated overnight at 4 $^{\circ}$ C with diluted primary antibody (*MUCDHL* H-225, 1/500; Santa Cruz Biotechnology). Cells were then washed 3 times with PBS 1X and

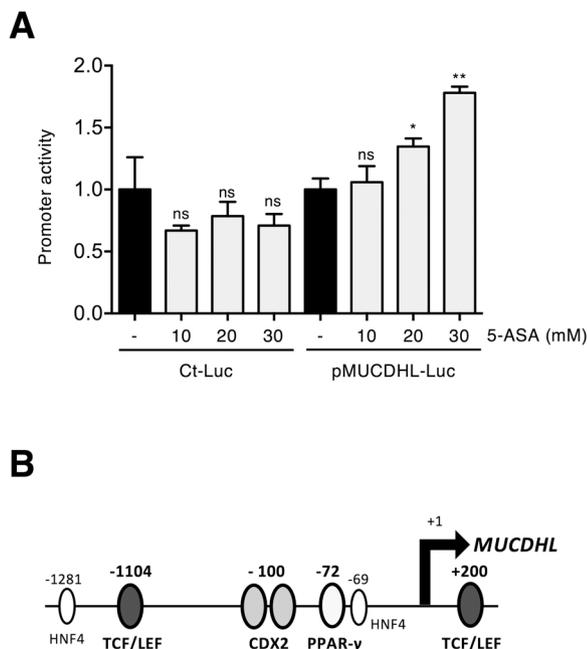


Fig. 2. Analyses of *MUCDHL* promoter. (A) Luciferase assay in SW480 cells transfected with control (Ct-Luc, pGL3-basic) or *MUCDHL* (pMUCDHL-Luc, pMUCDHL 1.4 kb-Luc) reporter plasmid and treated with increasing doses of 5-ASA for 24 h. Promoter activity corresponds to relative Luciferase activity (\pm SEM, normalized with renilla values). Values obtained for the different concentrations of 5-ASA were statistically compared to those of untreated cells set at 1 for each luciferase reporter plasmid ($n = 3$, Student *t*-test). (B) Schematic representation of the human *MUCDHL* promoter with the position of potential binding sites for transcription factors of interest. About 2000 bp of mouse and human genomic sequences around the transcription start were aligned with zPicture and conserved binding sites for transcription factors sought with rVista.

incubated for 45 min at room temperature with diluted secondary antibody (goat anti-rabbit IgG coupled with Alexa Fluor 488, 1/1000; Life Technologies). After washing with PBS, cells were stained with DAPI for 5 min at room temperature (1/25000, Sigma) and mounted with FluorSave Reagent (Calbiochem). Images were made using Axio Imager z2 (Zeiss).

2.10. Statistical analysis

Data from at least 3 independent experiments were compared using unpaired Student *t*-test (GraphPad Prism, version 6). Differences with $p > 0.05$ were considered as non-significant (ns), while differences with $p < 0.05$ (* or #), $p < 0.01$ (** or ##), $p < 0.001$ (***) or ####) and $p < 0.0001$ (**** or #####) were considered as significant.

3. Results

3.1. 5-ASA increases *MUCDHL* expression by stimulating its transcription

To extend the results obtained by Parenti and colleagues with Caco-2 cells [15], we first analyzed *MUCDHL* expression after mesalazine (hereafter called 5-ASA) treatment in a wider panel of human CRC cell lines. RT-qPCR experiments showed that the up-regulation of *MUCDHL* mRNA by 5-ASA occurs in most cell lines. Indeed, *MUCDHL* expression was stimulated in DLD-1, SW480, HT29 and Caco-2 cells but not in HCT116 cells (Fig. 1A-B and Fig. A2). Stimulation occurred at every time point tested, with a peak at 48 h for most cell lines. Consistently, we observed using immunofluorescence that *MUCDHL* protein levels were also increased by 5-ASA (Fig. 1C-D). To better understand how 5-ASA

stimulates *MUCDHL* expression, we performed a luciferase reporter assay on SW480 cells transfected with a luciferase reporter plasmid containing 1.4 kb of the *MUCDHL* promoter [25]. After 24 h of treatment with increasing 5-ASA concentrations, we observed a slight but dose-dependent, specific and significant increase in luciferase activity (e.g. $\times 1.9$ with $p < 0.01$ at 30 mM, Fig. 2A), indicating that 5-ASA up-regulates *MUCDHL* expression by stimulating its transcription. Therefore, we analyzed the nucleotide sequence of the *MUCDHL* promoter and found potential binding sites for several transcription factors (i.e. CDX2, HNF4 α , TCF/LEF, PPAR- γ) that are conserved between human and mouse (Fig. 2B).

3.2. β -catenin represses basal expression of *MUCDHL* and is cell-specifically targeted by 5-ASA

We noticed with interest the presence of two potential TCF/LEF (T cell factor/Lymphoid enhancer-binding factor) binding sites within the *MUCDHL* promoter (Fig. 2B, distal at -1104 bp and proximal at +200 bp). Indeed, the β -catenin/TCF4 complex is believed to repress *MUCDHL* expression in colon cancer cell lines [30] and β -catenin nuclear localization was shown to be decreased by 5-ASA [15]. Thus, we wondered if this molecular event contributes to *MUCDHL* up-regulation by 5-ASA.

First, to test if the decrease of β -catenin nuclear localization induced by 5-ASA actually correlates with a diminution of its transcriptional activity, we used the TopFlash luciferase reporter assay [27] in SW480 cells. Indeed, treatment with increasing doses of 5-ASA inhibited β -catenin transcriptional activity (Fig. 3A). Therefore, to check the importance of β -catenin in the regulation of *MUCDHL* expression, we transfected SW480 cells (which have high β -catenin protein levels upon APC loss of function mutation) [31,32] with anti- β -catenin siRNA in the absence or presence of 5-ASA. As expected, the inhibition of β -catenin (Fig. A3A) led to a strong increase in *MUCDHL* basal expression (Fig. 3B). Moreover, when β -catenin was inhibited, 5-ASA could not stimulate *MUCDHL* expression anymore. These results suggest that in SW480 cells, 5-ASA up-regulates *MUCDHL* by targeting its repression by β -catenin (Fig. 3B).

Since the β -catenin/TCF4 complex rather activates than represses transcription, we wondered if β -catenin was actually recruited to one of the two consensus TCF/LEF-binding sequences within the *MUCDHL* promoter. Therefore, we performed ChIP experiments and found that endogenous β -catenin is enriched at both binding sites on the *MUCDHL* promoter (Fig. 3C). Of note, a similar level of enrichment was observed on a TCF/LEF binding site of the *AXIN 2* promoter used as a positive control (data not shown). Importantly, the recruitment of β -catenin at the *MUCDHL* promoter decreased after 5-ASA treatment, especially at the proximal TCF/LEF binding site (Fig. 3C).

To confirm the results obtained in SW480 cells, we performed the same experiments in human colorectal cancer DLD-1 cells, which also exhibited robust stimulation of *MUCDHL* by 5-ASA. First, we aimed to verify that 5-ASA also inhibits β -catenin transcriptional activity in these cells. Surprisingly, the TopFlash luciferase reporter assay [27] revealed that β -catenin transcriptional activity was not affected by 5-ASA treatment in DLD-1 cells (Fig. 3D). Moreover, whether the cells were treated or not by 5-ASA, *MUCDHL* mRNA levels were strongly increased upon silencing of β -catenin (Fig. 3E and Fig. A3B), indicating that β -catenin exerts its repression in both conditions. Consistently, we detected β -catenin at the two TCF/LEF binding sites of the *MUCDHL* promoter in control and 5-ASA-treated DLD-1 cells by ChIP (Fig. 3F). Altogether, these results indicate that: i) basal expression of *MUCDHL* is repressed by β -catenin in both cell lines; ii) 5-ASA stimulates the expression of *MUCDHL* by cell-specific mechanisms that are not obligatory depending on β -catenin levels.

Finally, we checked the involvement of TCF4 in the regulation of *MUCDHL* expression as β -catenin is usually associated with this transcription factor. However, the silencing of *TCF4* by siRNA (Fig. A3C-D)

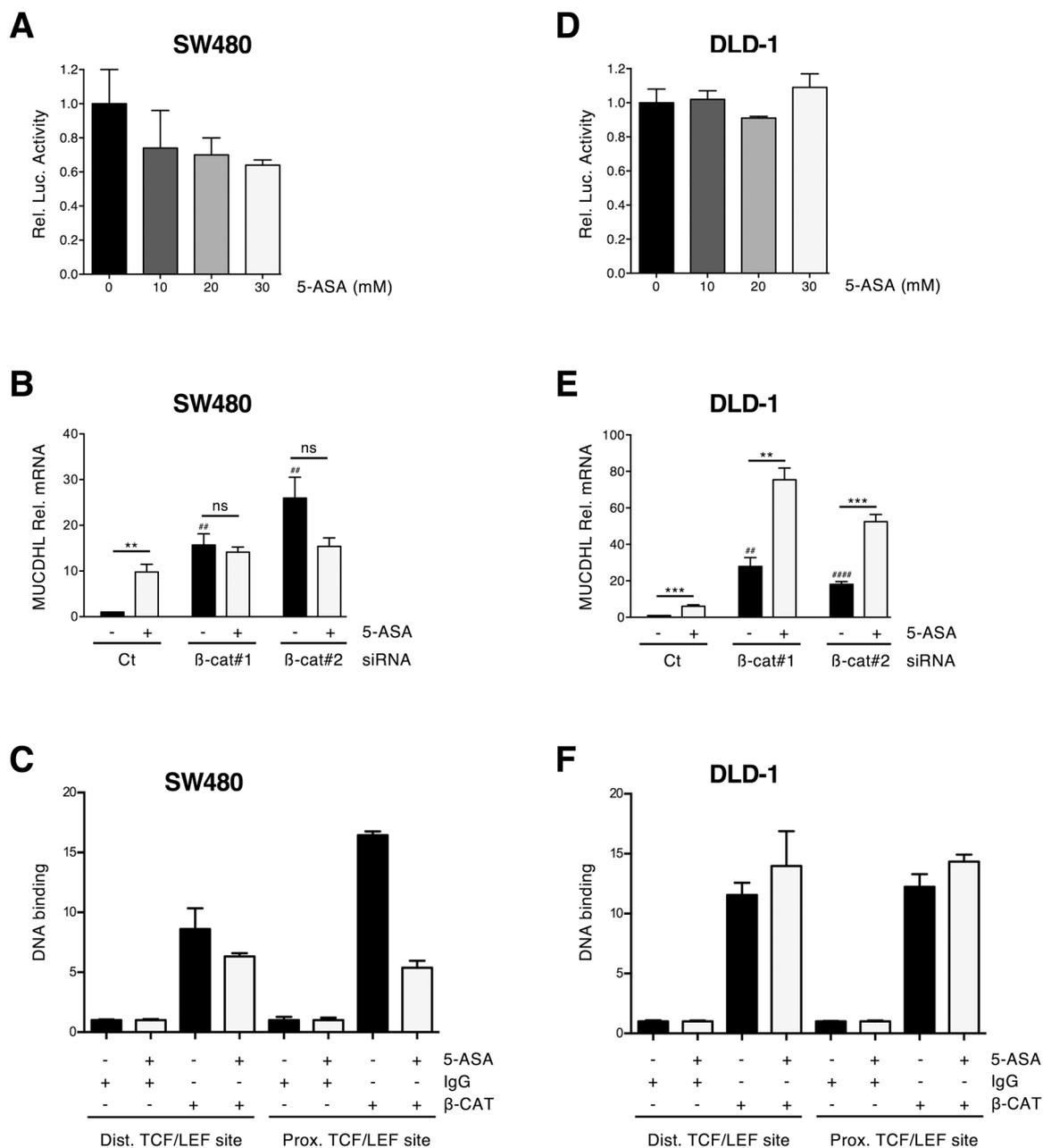


Fig. 3. Implication of β -catenin in basal and 5-ASA-stimulated *MUCDHL* expression. (A) Luciferase assay on SW480 cells transfected with the β -catenin /TCF TopFlash reporter plasmid along a promoter-less renilla reporter plasmid (internal control) and treated with increasing doses of 5-ASA for 24 h. Relative promoter activity (\pm SD, normalized with renilla values) of the TopFlash reporter in the absence of 5-ASA treatment was set at 1. (B) RT-qPCR analysis of SW480 cells transfected with the indicated siRNA (10 μ M) and treated or not with 5-ASA for 48 h. Relative mRNA levels of *MUCDHL* in untreated cells were set as 1 and data correspond to average RQ \pm SEM (n = 3, data were compared by Student *t*-tests with stars referring to treated vs untreated cells and hashtags to si β -cat vs siCt-transfected untreated cells). (C) Chromatin immunoprecipitation (ChIP) with control (IgG) or anti- β -catenin antibodies of SW480 cells treated or not with 5-ASA (48 h, 30 mM) followed by PCR amplification of the *MUCDHL* promoter fragments overlapping the distal or the proximal TCF/LEF binding site. DNA binding corresponds to enrichment compared to input and was set at 1 for values obtained with IgG. Data correspond to average RQ \pm SD of a representative experiment performed in triplicate. (D) same as (C) with DLD-1 cells (n = 4). (E) same as (B) for DLD-1 cells. (F) same as (E) for DLD-1 cells.

did not stimulate *MUCDHL* basal expression and did not prevent 5-ASA from stimulating *MUCDHL* expression in both cell lines (Fig. 4A-B). Moreover, ChIP experiments failed to detect strong binding of TCF4 to the *MUCDHL* promoter (Fig. 4C-D), even if it was clearly enriched at the *AXIN2* promoter (i.e. a well-known target gene) in the same extracts (data not shown). Thus, β -catenin is involved through TCF4-independent mechanisms in basal and 5-ASA-stimulated expression of *MUCDHL*.

3.3. | *CDX2* contributes to *MUCDHL* basal expression and is cell-specifically activated by 5-ASA

Following these unexpected results, we investigated whether other transcription factors are involved in *MUCDHL* up-regulation by 5-ASA in both cell lines. The homeotic transcription factor *CDX2* is a well-established regulator of *MUCDHL* basal expression in intestinal cells [25] and a publication suggested that *CDX2* may also be involved in *MUCDHL* stimulation by 5-ASA [30]. Therefore, we transfected SW480 and DLD-1 cells with either control or anti-*CDX2* siRNA (Fig. A3E-F) in

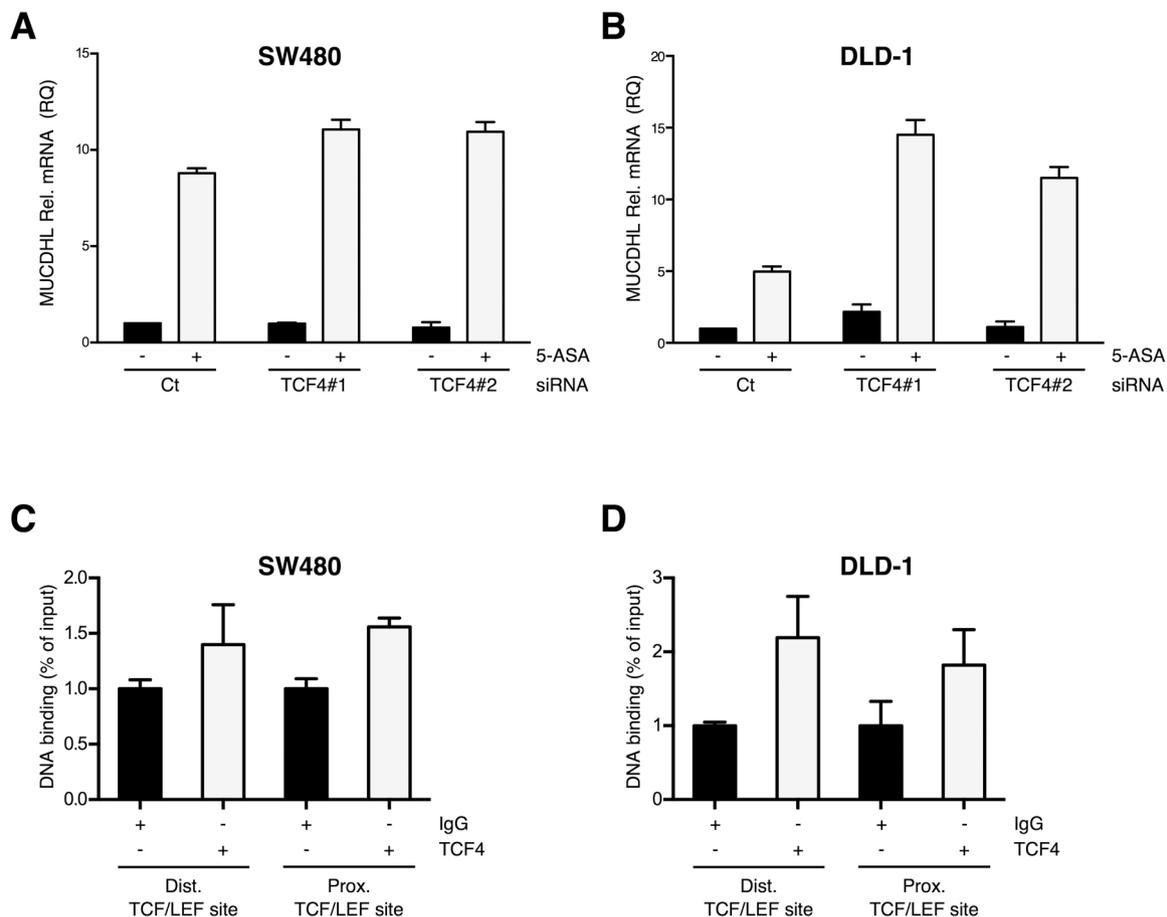


Fig. 4. Implication of TCF4 in *MUCDHL* expression. (A) RT-qPCR analyses of SW480 cells untreated or treated for 48 h with 30 nM of 5-ASA. Relative *MUCDHL* mRNA levels (normalized with *TBP* housekeeping gene) in untreated cells were set at 1 and data correspond to average RQ \pm SD ($n = 2$). (B) as (A) for DLD-1 cells. (C) Chromatin immunoprecipitation with control (IgG) or anti-TCF4 antibodies in SW480 cells followed by PCR amplification of the *MUCDHL* promoter fragments containing the distal (-1104 bp) or the proximal ($+200$ bp) TCF/LEF binding site. Data correspond to the average enrichments \pm SD of a representative experiment performed in triplicate. (D) as in (C) for DLD-1 cells.

the absence or presence of 5-ASA. As expected, *CDX2* inhibition decreased basal expression of *MUCDHL* in both cell lines (Fig. 5A-B). However, the robust stimulation of *MUCDHL* expression by 5-ASA treatment is observed in absence and in presence of *CDX2* in SW480 cells (Fig. 5A) but clearly blunted ($\sim 60\%$) by *CDX2* silencing in DLD-1 cells (Fig. 5B). We further confirmed the involvement of *CDX2* in these cells by ChIP experiments. Indeed, we observed an enrichment of *CDX2* to the *MUCDHL* promoter upon 5-ASA treatment (Fig. 5C). Taken together, these results confirm that 5-ASA stimulates *MUCDHL* expression by cell-specific mechanisms, with *CDX2*, in contrast to β -catenin, being a molecular effector of its activity in DLD-1 cells but not in SW480 cells.

3.4. PPAR- γ is a new 5-ASA-induced regulator of *MUCDHL* expression

As we noticed that *CDX2* silencing does not totally abolish the 5-ASA stimulation in DLD-1 cells, we hypothesized that another transcription factor may also be involved. Examination of the *MUCDHL* promoter revealed the presence of a conserved binding site for the transcription factor PPAR- γ , which belongs to the nuclear receptor superfamily (Fig. 2B). Interestingly, 5-ASA is known to increase the expression of PPAR- γ , but also its activity through direct fixation [33]. However, in contrast to *CDX2* and β -catenin, PPAR- γ has never been implicated in the regulation of *MUCDHL* expression. To test this hypothesis, we first used a PPAR- γ agonist: Troglitazone (TGZ) had no impact on *MUCDHL* expression in SW480 cells but increased it in DLD-1 cells, even if the stimulation was much weaker than the one obtained with 5-ASA (e.g. $\times 3$

with $p < 0.01$ at $50 \mu\text{M}$; Fig. A4A-B). To confirm these results, we overexpressed PPAR- γ by transfecting the cells with a pFlag-hPPAR- $\gamma 1$ plasmid (Fig. 6A-B): indeed, overexpression of PPAR- γ led to a stimulation of *MUCDHL* expression in DLD-1 cells ($\times 2.6$ with $p < 0.001$) but not in SW480 cells; moreover, when combined with 5-ASA treatment, PPAR- γ overexpression yielded an even stronger stimulation of *MUCDHL* expression in DLD-1 cells but not in SW480 cells. This result correlated with the increased nuclear localization of PPAR- γ specifically observed in 5-ASA-treated DLD-1 cells by sub-cellular fractionation (Fig. A4C-D).

To further prove the involvement of PPAR- γ , we inhibited its endogenous expression by siRNA (Fig. A3G-H). As expected, silencing of PPAR- γ did not change *MUCDHL* basal expression in either cell line (Fig. 6C-D). In contrast, it clearly lowered ($\sim 50\%$) the impact of 5-ASA on *MUCDHL* expression in DLD-1 cells but not in SW480 cells (Fig. 6C-D). We confirmed that PPAR- γ is directly involved in the regulation of *MUCDHL* expression in DLD-1 cells by performing ChIP experiments: we found that PPAR- γ was bound to the *MUCDHL* promoter (Fig. 6E) as efficiently as to one of its known target gene (Adipophilin, data not shown). In addition, this binding was increased by 5-ASA treatment ($\times 3$). Altogether, these results demonstrate that PPAR- γ is involved in *MUCDHL* stimulation by 5-ASA in DLD-1 cells.

As the individual silencing of PPAR- γ or *CDX2* only reduced the stimulation of *MUCDHL* expression by 5-ASA, we examined the consequences of silencing both of them. Strikingly, *MUCDHL* expression was not stimulated anymore by 5-ASA in DLD-1 cells simultaneously transfected by siRNA targeting PPAR- γ or *CDX2* (Fig. 6F). This strongly

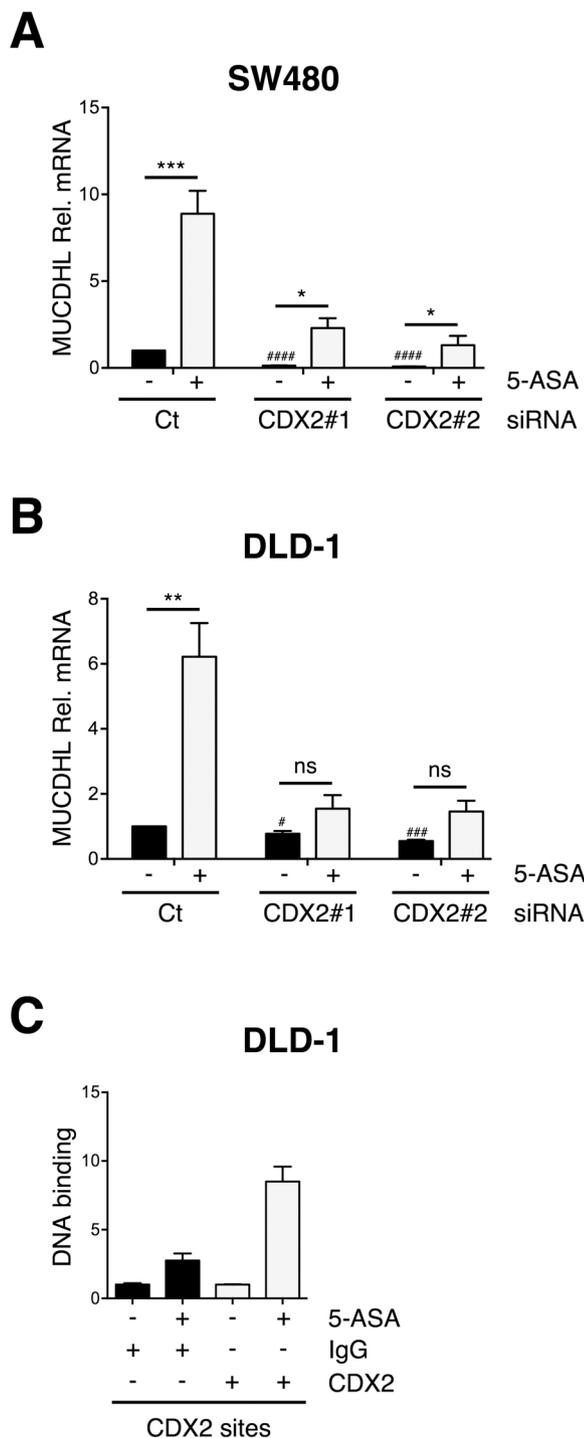


Fig. 5. Implication of CDX2 in basal and 5-ASA-stimulated *MUCDHL* expression. (A) RT-qPCR analysis of SW480 cells transfected with the indicated siRNA (10 μ M) and treated or not with 5-ASA (48 h, 30 nM). Relative mRNA levels of *MUCDHL* in untreated cells were set as 1 and data correspond to average RQ \pm SEM (n = 3, data were compared by Student *t*-tests with stars referring to treated vs untreated cells and hashtags to si-cat vs siCt-transfected untreated cells). (B) same as (A) with DLD-1 cells. (C) Chromatin immunoprecipitation (ChIP) with control (IgG) or anti-CDX2 antibodies of DLD-1 cells treated or not with 5-ASA (48 h, 30 nM) followed by PCR amplification of a *MUCDHL* promoter fragment containing CDX2 binding sites. DNA binding corresponds to enrichment compared to input and was set as 1 for values obtained with IgG. Data correspond to average RQ \pm SD of a representative experiment performed in triplicate.

suggests that these transcription factors are ensuring the impact of 5-ASA in DLD-1 cells.

3.5. 5-ASA increases the expression of genes involved in the intermicrovillar adhesion complex

Finally, we wondered if 5-ASA just stimulates *MUCDHL* expression or also other genes of the intermicrovillar adhesion complex (IMAC) linking microvilli together at the apex of absorbent cells [18]. RT-qPCR experiments revealed that, similarly to *MUCDHL*, 5-ASA also stimulates the expression of *CDHR2* and *MYO7B* in both SW480 and DLD-1 cells (Fig. 7A-B and Fig. A5A-D). By contrast, the mRNA expression of the adapter *USH1C* was only slightly increased by 5-ASA treatment in DLD-1 cells (Fig. A5E-F). Altogether, these results suggest that 5-ASA favors the assembly and maintenance of the brush border through the IMAC.

4. Discussion

Even if biologics have revolutionized the management of IBD, mesalazine remains the first-line treatment for mild to moderate UC. This well-tolerated and cost-effective 5-aminosalicylic acid compound does not only induce and maintain clinical remission but also enhances mucosal healing and has chemoprophylactic activity against CRC [9]. However, a clear understanding of the molecular mechanisms involved in these different activities is still lacking. Here, we investigated more thoroughly the regulation of *MUCDHL* by mesalazine in CRC cells as this gene might represent a critical node for its impact on cancer prevention and mucosal healing.

4.1. Different molecular mechanisms relay the impact of 5-ASA in CRC cells

As CRC is a heterogenous disease [31,34,35], we selected two CRC cell lines that showed robust 5-ASA-stimulation of *MUCDHL* expression and were suitable for gain / loss of function analyses to dissect the molecular mechanisms involved in the up-regulation of *MUCDHL*. These cell lines were established from colon tumors with different mechanisms of genetic instability, having either a chromosomal instability (CIN: SW480) or a microsatellite instability / CpG island methylator (MSI / CIMP: DLD-1) phenotype, and distinctive genetic alterations (e.g., *TP53*, *APC*, *KRAS* mutated in both cell lines but *PIK3CA* only mutated in DLD-1 cells) [31]. Unexpectedly, we found that 5-ASA stimulates *MUCDHL* expression by different mechanisms in the two cell lines: indeed, 5-ASA lifts off β -catenin repression in SW480 cells, while it activates CDX2 and PPAR- γ in DLD-1 cells to increase *MUCDHL* levels. The transcriptional regulators identified play essential roles in the intestinal epithelium and have been involved in both CRC and IBD [32,36-40]. The fact that 5-ASA is able to block the WNT/ β -catenin pathway is well documented: on the one hand, 5-ASA can induce phosphorylation of β -catenin at Threonine 41 and Serine 45 leading to its degradation by the proteasome, and on the other hand 5-ASA can decrease the transcriptional activity of β -catenin by inhibiting its phosphorylation at Serine 552 [41, 42]. Similarly, 5-ASA was shown to increase the activity of PPAR- γ by direct fixation [33]. Concerning CDX2, we observed an increase of its mRNA levels upon 5-ASA treatment in DLD-1 cells (data not shown) that may at least in part explain its increased activity. However, beyond the control of *MUCDHL* expression, our data highlight the cell-specific effects of 5-ASA: indeed, we showed that 5-ASA does not inhibit the transcriptional activity of β -catenin in DLD-1 cells and rather suppresses PPAR- γ activity in SW480 cells, while having no effect on *MUCDHL* expression in HCT116 cells. Differences in genetic backgrounds (e.g. *TP53* mutated in SW480 and DLD-1 cells but wild-type in HCT116 cells and the opposite for *CTNBN1*; *PIK3CA* mutated in HCT116 cells and DLD-1 cells and wild-type in SW480 cells) [31] and expression levels of the different factors between the cell lines are likely to be at the root of

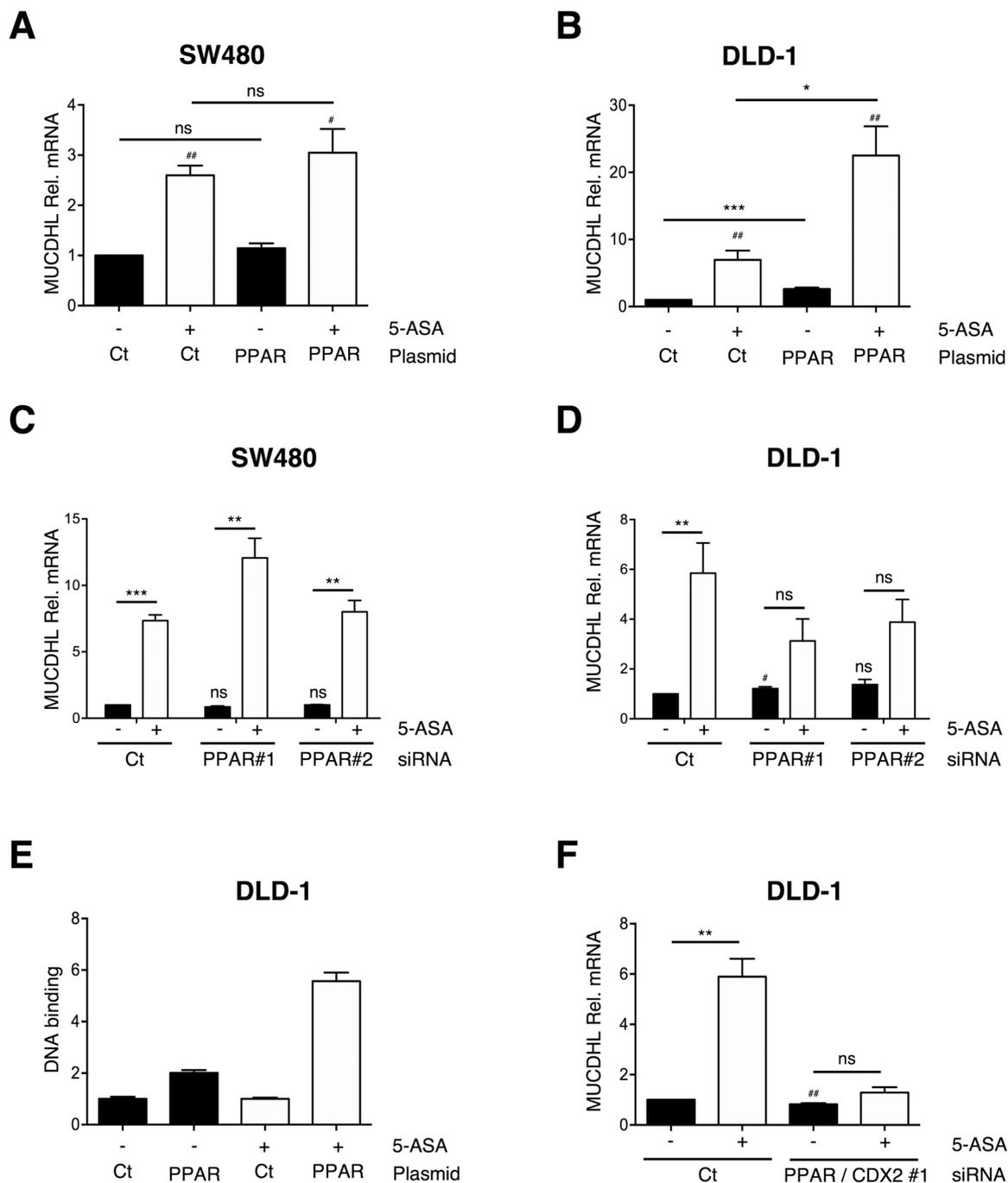


Fig. 6. Implication of PPAR- γ in 5-ASA-stimulation of *MUCDHL* expression. (A) RT-qPCR analysis of SW480 cells transfected with control (Ct) or FLAG-PPAR- γ -expressing (PPAR) plasmid and treated or not with 5-ASA (48 h, 30 nM). Relative mRNA level of *MUCDHL* in untreated cells transfected with control plasmid was set at 1 and data correspond to average RQ \pm SEM (n = 3, data were compared by Student *t*-tests with hashtags referring to treated vs untreated cells and stars to PPAR- γ vs Ct plasmid transfected cells). (B) same as (A) with DLD-1 cells (n = 4). (C) RT-qPCR analysis of SW480 cells transfected with the indicated siRNA (10 μ M) and treated or not with 5-ASA (48 h, 30 nM). Relative mRNA levels of *MUCDHL* in untreated cells were set as 1 and data correspond to average RQ \pm SEM (n = 3, data were compared by Student *t*-tests with stars referring to treated vs untreated cells and hashtags to siCt vs siCt-transfected untreated cells). (D) same as (C) with DLD-1 cells. (E) Chromatin immunoprecipitation (ChIP) with anti-FLAG antibodies of DLD-1 cells transfected with control (Ct) or FLAG-PPAR- γ -expressing (PPAR) plasmid and treated or not with 5-ASA (48 h, 30 nM), followed by PCR amplification of a *MUCDHL* promoter fragment containing a PPAR- γ - binding site. DNA binding corresponds to enrichment compared to input and was set at 1 for values obtained with control plasmid-transfected cells. Data correspond to average RQ \pm SD of a representative experiment performed in triplicate. (F) As in (D) but DLD-1 cells were co-transfected with a mix of PPAR and CDX2 siRNA (10 μ M total) or control siRNA (10 μ M).

the cell-specific effect of 5-ASA, especially as molecular interactions and cross-talks between β -catenin, CDX2 and PPAR- γ have been reported [43–46]. Anyhow, our results suggest that the molecular heterogeneity of CRC cell lines or even neoplastic cells in patients may explain some of the discrepancies between different studies about 5-ASA and may

provide a rationale for patient stratification.

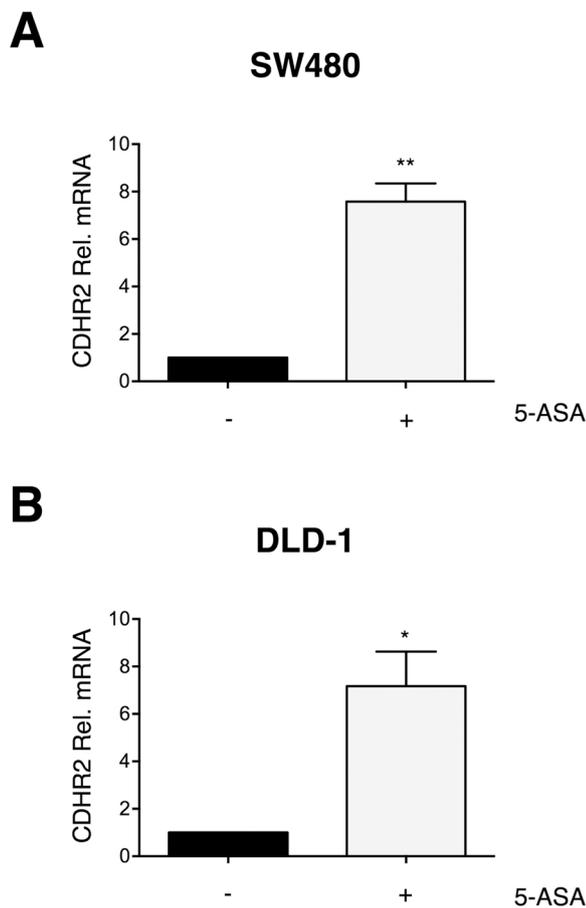


Fig. 7. Impact of 5-ASA on *CDHR2* expression in CRC cell lines. (A) RT-qPCR analyses of SW480 cells untreated (-) or treated (+) with 30 mM of 5-ASA for 48 h. *CDHR2* mRNA level (normalized with those of a housekeeping gene) in untreated cells was set at 1 for untreated cells and data correspond to average $RQ \pm SEM$ ($n = 3$, untreated and treated cells were statistically compared using a Student *t*-test). (B) same as (A) with DLD-1 cells.

4.2. MUCDHL is emerging as an attractive 5-ASA target for mucosal healing and chemoprevention in IBD

Alteration of intestinal architecture associated with barrier dysfunction is a hallmark of IBD [47]. Mucosal healing is currently considered as a treatment end-point for UC as it lowers clinical relapse, hospitalization and surgery [48]. While 5-ASA has been shown to enhance mucosal healing, the underlying mechanisms of this effect have been poorly investigated. A recent study indicated that 5-ASA decreases intestinal permeability and leads to E-cadherin redistribution at the cell membrane [13]. MUCDHL might also be involved in the restoration of the barrier function by 5-ASA. Indeed, MUCDHL favors membranous localization of β -catenin and strengthens intercellular adhesion similarly to 5-ASA [15,16,23,49]. Furthermore, MUCDHL promotes the formation and stability of the intestinal brush border by mediating intermicrovillar adhesion [18]. The fact that 5-ASA also stimulates the expression of MUCDHL partners within the IMAC (i.e. *CDHR2* and *MYO7B*) suggests for the first time an impact on the brush border itself that might be critical for mucosal healing. Interestingly, microvilli defects (i.e. length and ultrastructure) associated with reduced levels of IMAC genes were recently described in uninflamed intestinal tissues from IBD patients and were shown to correlate with a more severe disease [21]. While the consequences of these alterations in the context of chronic inflammation need to be further investigated, several studies have shown that MUCDHL and *CDHR2* are critical for intestinal homeostasis and oppose tumorigenesis [24,25,50,51]. Gut leakiness is associated with

microbiota dysbiosis, low grade immune activation and inflammation, which contribute to tumorigenesis [52,53]. Hence, the ability of 5-ASA to restore intestinal permeability may be highly meaningful for its chemoprophylactic activity against CAC. Furthermore, both *CDHR2* and *MUCDHL* inhibit the transcriptional activity of oncogenic β -catenin [24, 54]. As *MUCDHL* is involved in a negative feedback loop with nuclear β -catenin [25,30], its up-regulation by 5-ASA is predicted to reinforce the inhibition of the transcriptional activity of β -catenin and thereby dampen stemness and proliferation of intestinal cells in areas that regenerate post-inflammation [26,32].

4.3. Limitations of the study

This study was performed on cell lines established from sporadic CRC and will require further validation in conditions that better reflect the pathogenesis of CAC. Its findings highlight context-dependent molecular responses to 5-ASA treatment whose determinants remain to be explored. Thus, while providing novel results regarding the mechanisms of *MUCDHL* regulation by 5-ASA, it opens new avenues for additional *in vivo* research in IBD patients in active or remission phase of the disease and in associated cancers.

5. Conclusion

In summary, this study sheds a new light on the molecular mechanisms involved in the epithelial activity of 5-ASA, thereby providing a rationale for its long-term use for UC and potential biological markers to improve disease management.

CRediT authorship contribution statement

EB, CT, ML, EM, CQ and IG performed the research; EB, CT, ML, CQ, JNF, JMR, and IG collected and analyzed the data; EB, CT, JMR and IG designed the research study and wrote the paper, and JNF contributed to the design of the study.

Conflict of interest statement

JMR declares consulting fees from Hospira, lecture fees from Abbvie, Ferring, Janssen, Pfizer and Takeda and travel accommodations from Ferring, Abbvie, MSD, Janssen, Pfizer, Hospira and Takeda but this did not impact the study. Therefore the authors declare that there are no conflicts of interest regarding the publication of this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2021.112543](https://doi.org/10.1016/j.biopha.2021.112543).

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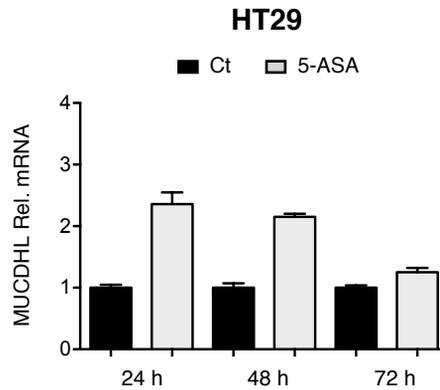
SUPPLEMENTARY FIGURE A1

Fragment	Forward primer	Reverse primer
MUCDHL (PPAR- γ /CDX2 sites)	GCTGGACCAGCCGGCCAGCG	TCTTGGCGGCTGTCACCTGGCA
MUCDHL (Prox TCF4 site)	CTTACAGACTGCTCTGTGA	ATCAGGAGTCACGTTGAGA
MUCDHL (Dist TCF4 site)	TACCTCGACTAAGTACTGCA	CAGAGTGGATTCTGGCAC
ADIPOPHILIN (PPAR- γ site)	AGAAGCTTGCTCAGCCTTAAGTGA	CCTGCTCTAATGACCTCTTAC
SUCRASE-ISOMALTASE (CDX2 site)	GGCTGGTAAGGGTGCAATAA	GCCTGTTCTCTTTGCTATGTTG
AXIN 2 (TCF4 site)	CTGGAGCCGGCTGCGCTTTGATAA	CGGCCCCGAAATCCATCGCTCTGA

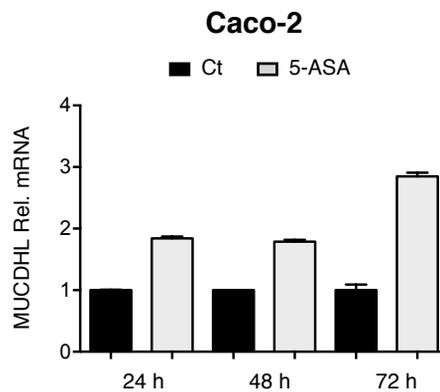
Supplementary Fig. A1. Primer sequences used for ChIP experiments. Adipophilin, Sucrase-Isomaltase and Axin 2 were used as positive controls.

SUPPLEMENTARY FIGURE A2

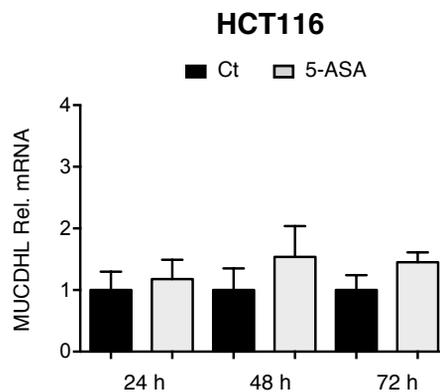
A



B

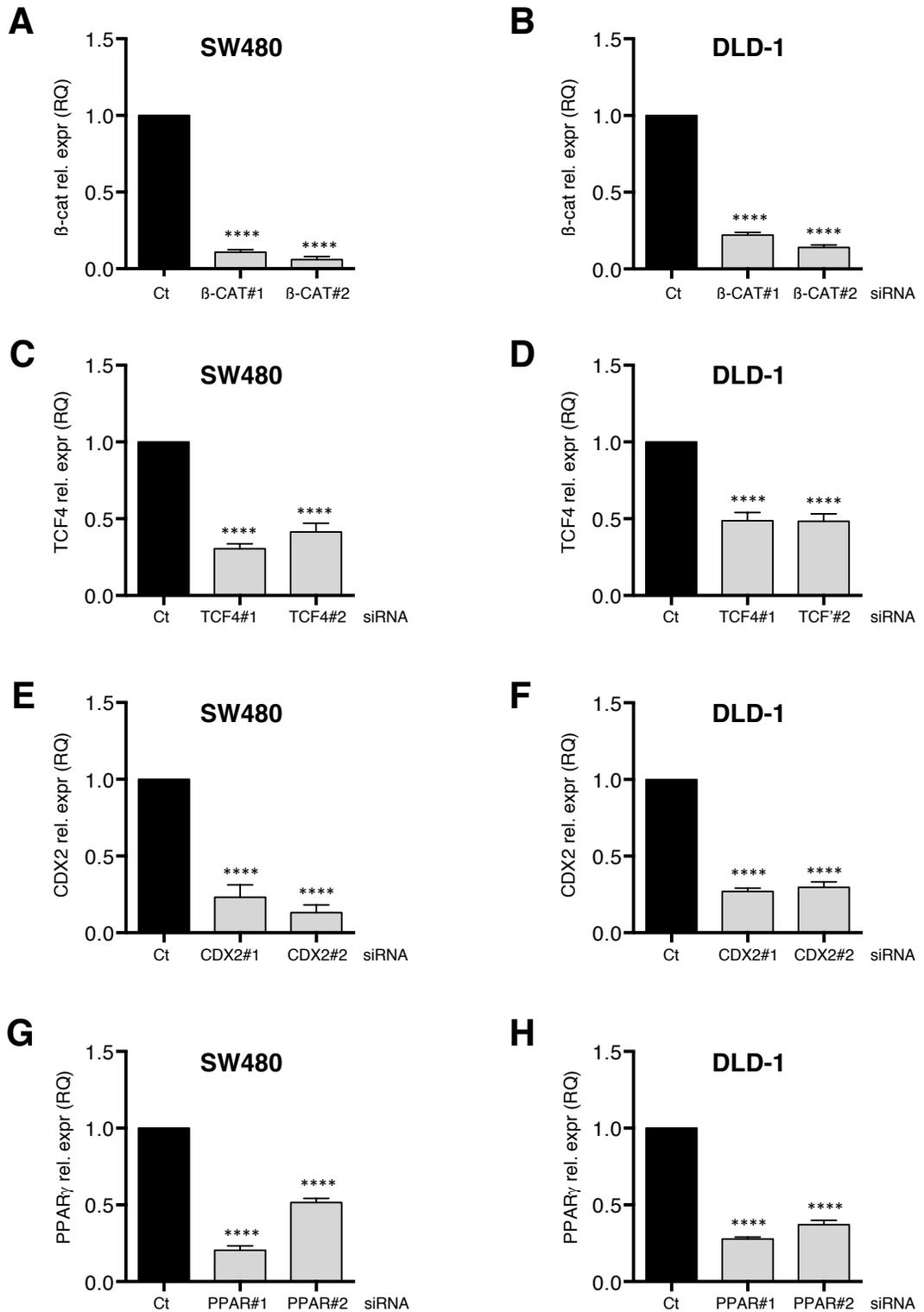


C



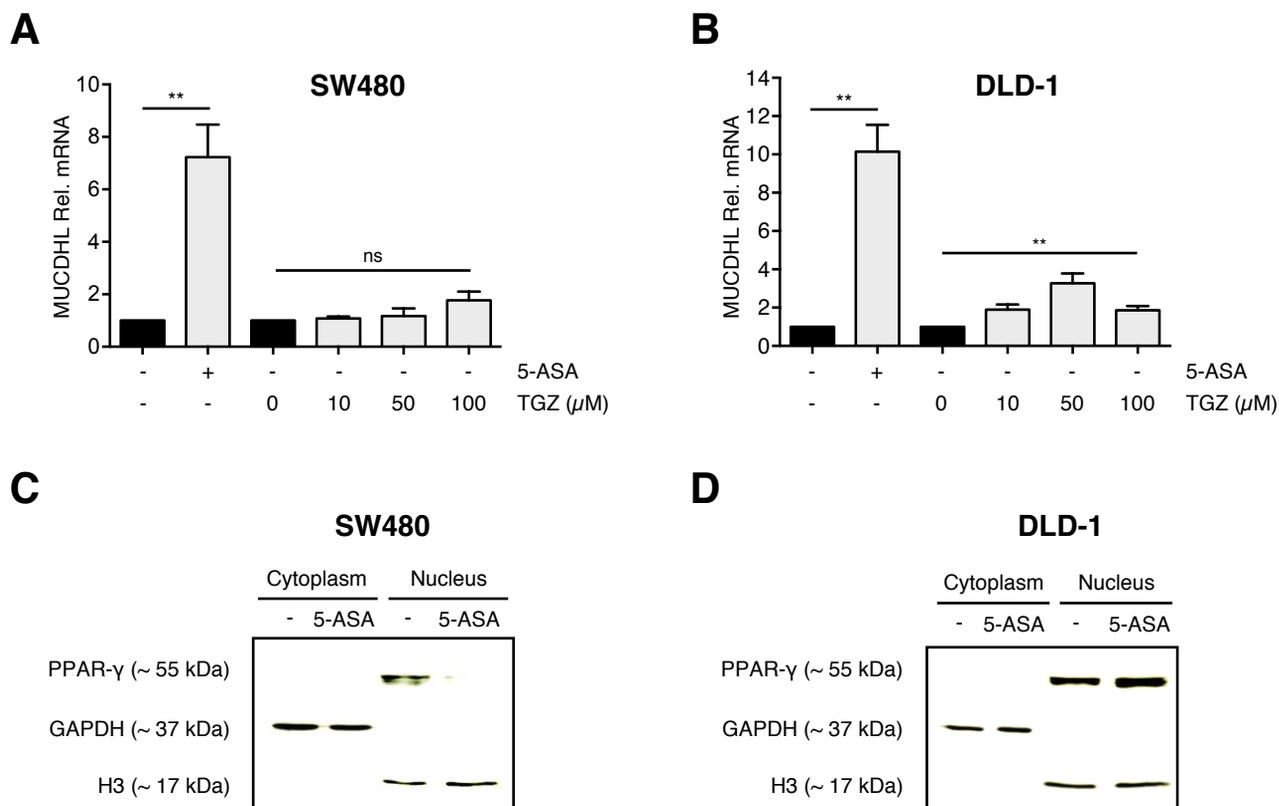
Supplementary Fig. A2. Impact of 5-ASA on *MUCDHL* expression in CRC cell lines. (A) RT-qPCR analyses of HT29 cells untreated or treated for the indicated time with 30 nM of 5-ASA. Relative *MUCDHL* mRNA levels (normalized with *TBP* housekeeping gene) in untreated cells were set at 1 and data correspond to average RQ \pm SD of a representative experiment. (B) Same as in (A) for Caco-2 cells. (C) Same as in (A) for HCT116 cells.

SUPPLEMENTARY FIGURE A3



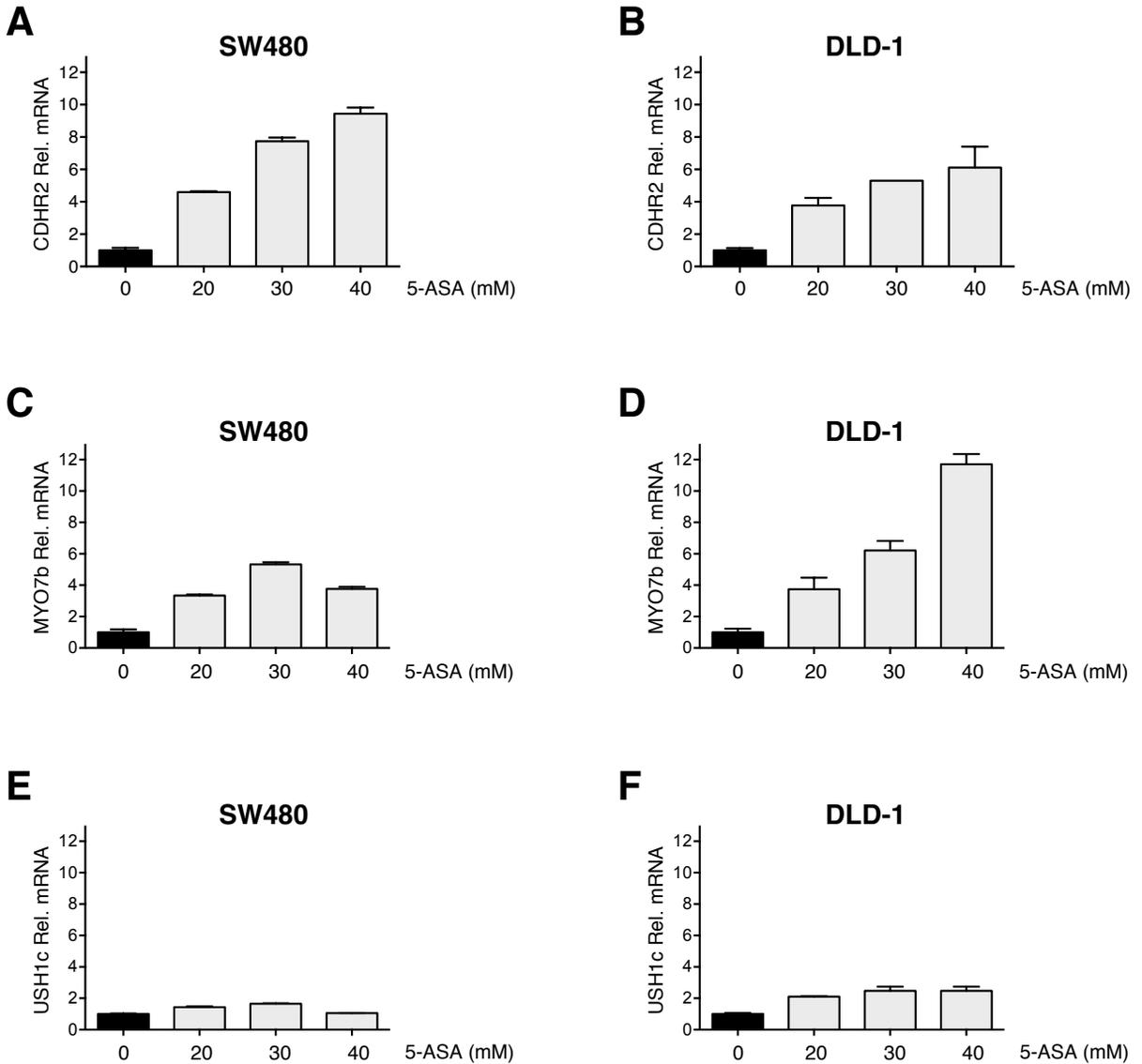
Supplementary Fig. A3. Efficacy of siRNA silencing. Relative mRNA expression of β -catenin, *TCF4*, *CDX2*, and *PPAR- γ* after 48 h of transfection with control (siCt) or specific (#1 and #2) siRNA (10 nM) was determined by RT-qPCR. Expression (normalized with *TBP* housekeeping gene) in cells transfected with siCt was set as 1. Data correspond to means \pm SEM of 3-7 independent experiments (***) $p < 0.0001$ compared to siCt-transfected cells and calculated with a Student t test).

SUPPLEMENTARY FIGURE A4



Supplementary Fig. A4. Implication of PPAR- γ in *MUCDHL* expression. (A-B) Impact of a PPAR- γ agonist on *MUCDHL* expression. (A) RT-qPCR analysis of SW480 cells untreated or treated for 48 h with 5-ASA (30 mM) or increasing concentrations of Troglitazone (TGZ). Relative *MUCDHL* mRNA levels (normalized with *TBP* housekeeping gene) in untreated cells were set at 1. Relative mRNA levels of *MUCDHL* in untreated cells were set at 1 and data correspond to average RQ \pm SEM ($n = 3$, data were compared by Student t tests with stars referring to treated vs untreated cells) (B) Same as in (A) for DLD-1 cells. (C-D) Impact of 5-ASA on the sub-cellular localization of PPAR- γ . (C) Western blot detection of PPAR- γ in cytoplasmic and nuclear extracts of SW480 cells treated or not with 30 mM 5-ASA for 6 h. Detection of GAPDH (cytoplasmic) and H3 (nuclear) were performed to check the efficiency of sub-cellular fractionation. (D) same as in (C) for DLD-1 cells.

SUPPLEMENTARY FIGURE A5



Supplementary Fig. A5. Impact of 5-ASA on IMAC genes in CRC cell lines. (A-B) RT-qPCR analyses of (A) SW480 cells and (B) DLD-1 cells untreated (0) or treated with the indicated concentration of 5-ASA for 48 h. *CDHR2* mRNA levels (normalized with those of a housekeeping gene) in untreated cells were set at 1 for each concentration and data correspond to average RQ \pm SD of a representative experiment performed in triplicate. (C-D) Same as in (A-B) for *MYO7b*. (D-E) Same as in (A-B) for *USH1c*.