

# KLF4 Mediates the Effect of 5-ASA on the $\beta$ -Catenin Pathway in Colon Cancer Cells

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## Abstract

Mesalazine (5-ASA) is an aminosalicilate anti-inflammatory drug capable of inducing  $\mu$ -protocadherin, a protein expressed by colorectal epithelial cells that is downregulated upon malignant transformation. Treatment with 5-ASA restores  $\mu$ -protocadherin expression and promotes the sequestration of  $\beta$ -catenin to the plasma membrane. Here, we show that 5-ASA-induced  $\mu$ -protocadherin expression is directly regulated by the KLF4 transcription factor. In addition, we suggest the existence of a dual mech-

anism whereby 5-ASA-mediated  $\beta$ -catenin inhibition is caused by  $\mu$ -protocadherin-dependent sequestration of  $\beta$ -catenin to the plasma membrane and by the direct binding of KLF4 to  $\beta$ -catenin. In addition, we found that 5-ASA treatment suppresses the expression of miR-130a and miR-135b, which target KLF4 mRNA, raising the possibility that this mechanism is involved in the increased expression of KLF4 induced by 5-ASA. *Cancer Prev Res*; 11(8); 503–10. ©2018 AACR.

## Introduction

Colorectal cancer arises as a result of a multistep process in which well-characterized genetic and epigenetic alterations, causing the activation of proto-oncogenes and the inactivation of tumor-suppressor genes, occur in a sequential order (1). Chemoprevention with Non-Steroidal Anti-Inflammatory Drugs (NSAID) is a strategy currently used to inhibit the development of colorectal cancer in patients with pre-malignant conditions (2); however, the systemic and gastrointestinal toxicity of these compounds drastically limits their administration in clinical protocols requiring long-term treatments. A number of drugs characterized by a similar pharmacological activity, but devoid of the severe side

effects of NSAIDs, are currently under investigation; one such drug is Mesalazine (5-Aminosalicylic Acid or 5-ASA; ref. 2) that exerts its chemopreventing effect by inhibiting the  $\beta$ -catenin signaling pathway, resulting in reduced proliferation and increased differentiation. These effects were observed in different tumor types, including colorectal cancer (3, 4). The molecular mechanisms underlying 5-ASA action are not clearly understood. In the past few years, our research group has contributed to clarify this issue demonstrating the involvement of a protein named  $\mu$ -protocadherin, that is encoded by the *MUCDHL* gene. The expression of this protein is silenced during colorectal cancer carcinogenesis (5, 6) but is upregulated upon treatment with 5-ASA and acts by sequestering  $\beta$ -catenin to the plasma membrane. Additional experiments performed in our laboratory indicate that the expression of  $\mu$ -protocadherin is negatively regulated by  $\beta$ -catenin, suggesting that a mechanism of reciprocal inhibition regulates the activity of these two proteins (7).

Kruppel-like factor 4 (KLF4) is a transcription factor highly expressed in the adult intestine (8) and is critically important in the process of differentiation (9). KLF4 interacts with  $\beta$ -catenin and inhibits Wnt signaling (10). Given the critical role of  $\beta$ -catenin in mediating Wnt signaling and in the development of colorectal cancer, a better understanding of the mechanism of KLF4-mediated inhibition may lead to novel therapies for colorectal cancer. However, the precise molecular mechanisms of how KLF4 inhibits  $\beta$ -catenin are not entirely clear.

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In this study, we further analyzed the chemopreventing role of 5-ASA. Our data suggest that this compound is capable of inducing KLF4 expression, which, in turn, inhibits Wnt/ $\beta$ -catenin signaling by preventing the interaction of  $\beta$ -catenin with TCF4 and by directly inducing  $\mu$ -protocadherin expression leading to  $\beta$ -catenin sequestration to the plasma membrane.

## Materials and Methods

### Microarray analysis

We analyzed the expression of KLF4-induced genes in untreated and 20 mmol/L 5-ASA-treated CaCo2 cells, as previously described (6), using the Affymetrix Human HG-U133 plus2 GeneChip array, according to Affymetrix standard protocols (Thermo Fisher Scientific; ref. 11).

### Cell lines

CaCo2, HT29, and HEK293T cell lines were obtained from the ATCC and cultured in DMEM medium (Euroclone), supplemented with 10% heat-inactivated FBS (Sigma-Aldrich) and 1 mmol/L L-Glutamine (Euroclone).

5-ASA (SofarFarm S.p.A.) was dissolved in complete DMEM medium at the concentration of 10 to 20 mmol/L and experiments were performed protected from light.

### Protein extracts preparation and Western blot analysis

Total extracts of CaCo2 cells were obtained as previously reported (12). Briefly, 50  $\mu$ g of protein extracts were loaded onto 10% SDS-polyacrylamide gel and blotted as described (13). Membranes were pre-blocked in blocking solution, supplemented with 5% non-fat milk (Regilait), for 1 hour at room temperature and then incubated with the appropriated primary antibody diluted according to the manufacturer's protocol, followed by a 1-hour incubation at room temperature with a specific secondary antibody conjugated to horseradish peroxidase (HRP). The following primary antibodies and the respective dilutions were used for Western blot analysis: mouse anti- $\mu$ -protocadherin monoclonal antibody (MoAb; A-11, Santa Cruz Biotechnology, Inc.) 1:500 in TBST 3% milk; rabbit anti-KLF4 MoAb (Abcam) 1:1,000 in TBST 1% milk; rabbit anti-TCF4 MoAb (Cell Signaling Technology, Inc.) 1:1,000 in TBST 5% BSA. Expression of vinculin was also evaluated with a mouse anti- $\alpha$ -vinculin MoAb (Millipore Corporation) to normalize the protein content of the various analyzed samples. As secondary antibodies, a goat anti-mouse IgG (Santa Cruz Biotechnology) or a goat anti-rabbit IgG (Cell Signaling Technology), both conjugated with HRP, were used respectively at 1:10,000 and 1:15,000 dilutions. Detection of Western blot signals was carried out using the Westar EtaC enhanced chemi-luminescent substrate (Cyanagen S.r.l.).

### Quantitative real-time PCR

Total RNA was extracted from the various analyzed cell populations by means of the Qiagen total RNA purification kits as recommended by the manufacturer (Qiagen) and RNA concentration was verified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Total RNA (100 ng) was reverse transcribed using High Capacity cDNA Archive Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time PCR (QRT-PCR) was then performed with an ABI PRISM 7900 sequence detection system using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) to quantify the relative levels of mRNA in the samples, as previously described (14). Evaluation of QRT-PCR signals was eventually carried out with the  $\Delta\Delta C_t$  relative quantification method, using the *GAPDH* gene to normalize analyzed samples. Thermo Fisher Scientific designed also primers and probe for KLF4,  $\mu$ -protocadherin, p21<sup>waf-1</sup>, CDX2 and GAPDH amplification. Pairwise comparison between 5-ASA-treated samples transfected with KLF4 siRNA and control siRNA and between 5-ASA-treated and -untreated samples transfected with control siRNA, were conducted using the Student's t-test procedure.

### siRNA transfection

Experiments of mRNA silencing were conducted transfecting a mix of three different siRNA oligonucleotides directed against KLF4 (Sigma-Aldrich) at a concentration of 100 nmol/L with RNAiMAX reagent (Thermo Fisher Scientific). The transfection efficiency of CaCo2 cells was potentiated using the reverse transfection procedure according to the manufacturer's instructions.

### Plasmid expression vector

A full-length KLF4 cDNA was generated by RT-PCR performed on total RNA extracted from CaCo2 cell line, using KLF4 DP (5'-ACATTAATGAGGCAGCCACC-3') and KLF4 RP (5'-TTCTGGCAGTGTGGGTCATA-3') primers. Amplification was carried out using a proofreading thermostable DNA polymerase (Roche Diagnostics S.p.A.) and the amplified fragment was inserted in the pCR2.1 T/A cloning vector (Thermo Fisher Scientific) where it was fully sequenced to exclude polymerase-induced mutations. KLF4 cDNAs were then excised with EcoRI enzyme and cloned in the EcoRI site of pcDNA3.1 plasmid (Thermo Fisher Scientific) resulting in pcDNA3.1-KLF4 construct. The pT81Luc vectors (15), containing KLF4 responsive elements (KRE), were generated as described below. Modified versions of the following single-strand oligomers: KRE1 probe 5'-TAGTGGGGAGGGTGTGAA-GG-3'; KRE2 probe 5'-GGGAGGGAGGTGCTCCCTGG-3', obtained by adding digested BamHI and Sall sequences at their ends, were annealed to their complementary strands, similarly projected. The resulting double-strand overhang oligomers were then inserted into the BamHI/Sall digested pT81Luc vector, upstream to a minimal

promoter and the luciferase reporter gene as previously described (16). According to these cloning strategies, the following constructs were generated: pT81LucKRE1, pT81LucKRE2. Nucleotide sequence analysis of the plasmids demonstrated that binding sequences had been inserted as single-copy oligomer.

#### Luciferase reporter assay

Luciferase transactivation assays were carried out in HEK293T cells as already described (17, 18). Transient transfection was conducted with Lipofectamin 2000 plus (Thermo Fisher Scientific), using 200 ng of pT81Luc reporter plasmids, 50 ng of pcDNA3.1-KLF4, 200 ng of pCMV $\beta$ -galactosidase (Clontech Laboratories, Inc.) to normalize for transfection efficiency, and a carrier plasmid to maintain a total DNA concentration of 800 ng. After a 48-hour incubation, cells were lysed according to standard protocols and  $\beta$ -galactosidase and luciferase enzymatic assays were performed.

TOPFlash Assays were carried out in CaCo2 cells, using TCF Reporter Plasmid Kit (Addgene). For these transfections, Lipofectamine 2000 plus (Thermo Fisher Scientific) was used as a transfection reagent with 250 ng of TOPFlash or FOPFlash reporter plasmids, 250 ng of pcDNA3.1-KLF4, 250 ng of pCMV $\beta$  galactosidase (Clontech Laboratories) and 20 mmol/L 5-ASA (SofarFarm S.p.A) were used.

#### Coimmunoprecipitation assay

CaCo2 cells were treated with 20 mmol/L 5-ASA and, after 96 hours, harvested to extract proteins with PBSTDS buffer as previously reported<sup>6</sup>. 300  $\mu$ g of total extract were incubated overnight at 4°C with 1 volume of HNTG Buffer, 30  $\mu$ L of Protein G Agarose (KPL, Gaithersburg, MD) and 2  $\mu$ g of mouse monoclonal antibody anti- $\beta$ -catenin (BD Biosciences) or rabbit polyclonal anti-KLF4 (AbCam). Anti-Mouse or anti-Rabbit-IgG (Santa Cruz Biotechnology) were used as negative controls. Beads were extensively washed with HNTG Buffer and samples were loaded onto 10% SDS-polyacrylamide gel electrophoresis. Immunoblot analysis was performed with mouse polyclonal antibody anti-TCF4 and with rabbit polyclonal antibody anti-KLF4.

#### miRNA expression assay

CaCo2 cells were treated with 20 mmol/L 5-ASA for 96 hours, harvested and total RNA, including small RNAs, was extracted with the mirVana miRNA Isolation Kit (Thermo Fisher Scientific). 500 ng of total RNA was reverse transcribed using Megaplex Pool For microRNA Expression Analysis (Thermo Fisher Scientific) as recommended by the manufacturer. The quantitation of 754 human microRNAs was then performed with TaqMan Array Human MicroRNA A+B Cards Set v3.0 (Thermo Fisher Scientific) on ABI PRISM 7900 HT

sequence detection system (Thermo Fisher Scientific), according to the manufacturer's protocol.

#### miRNA inhibitor transfection

100  $\mu$ mol/L of the following mirVana miRNA Inhibitors: Negative Control #1, miR130a, miR135b and miR130a and miR135b together, were transfected with Lipofectamine 2000 plus (all Thermo Fisher Scientific) for 48 hours according to the manufacturer's instructions.

## Results

### Treatment with 5-ASA induces KLF4 and $\mu$ -protocadherin expression

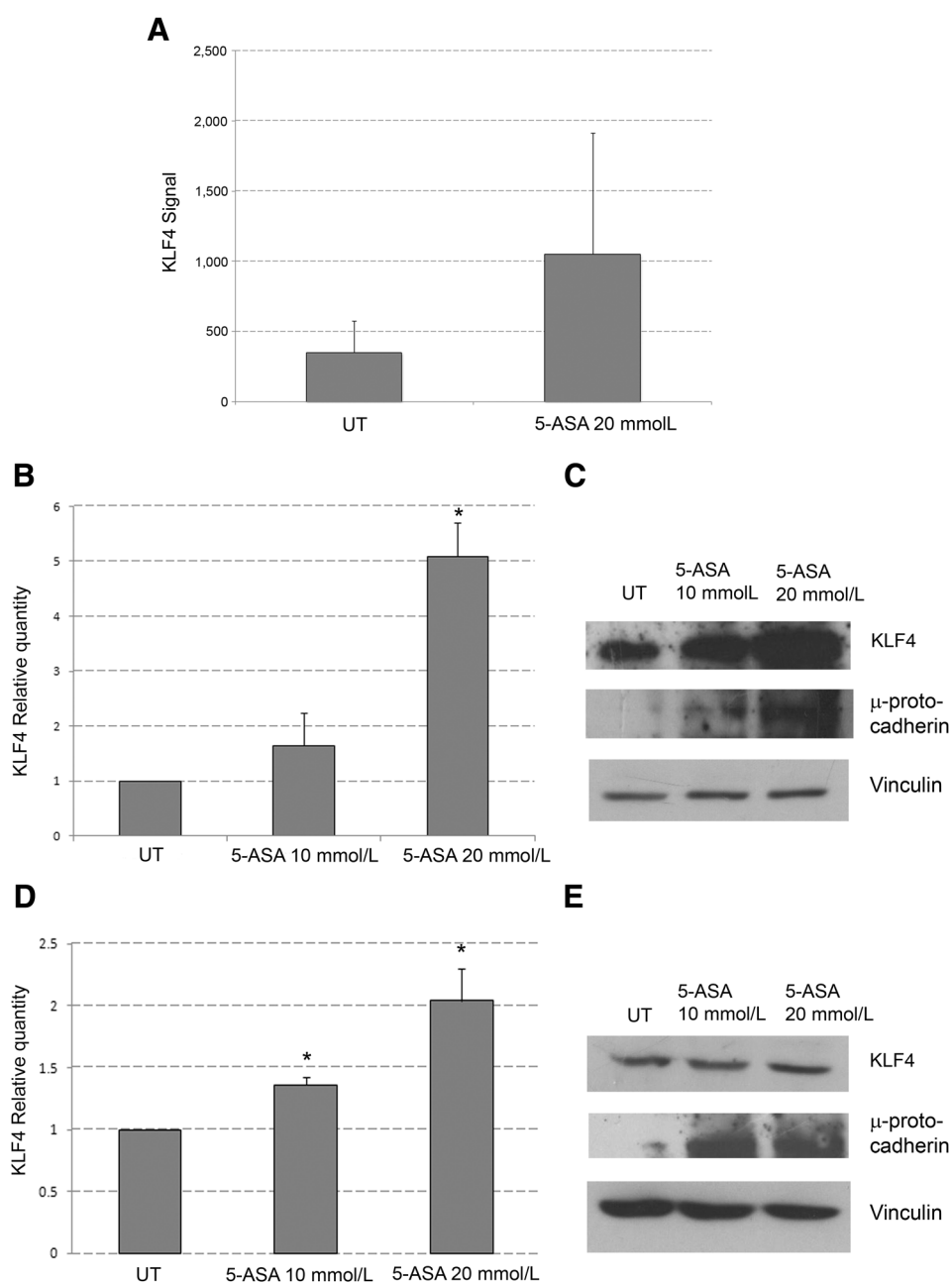
Preliminary findings suggesting that 5-ASA might induce KLF4 expression derive from microarray data of CaCo2 cells treated with 20 mmol/L 5-ASA (6). The increase in KLF4 signal is shown in Fig. 1A. Because these data were not statistically significant, they had to be further validated. To do so, the effect of 5-ASA on KLF4 expression in CaCo2 colorectal cancer cells line was assessed by QRT-PCR and Western blot. Fig. 1B shows that, compared with untreated cells, treatment with 5-ASA up-regulates KLF4 mRNA levels in a dose-dependent manner. Western blot analysis demonstrated that treatment with 20 mmol/L 5-ASA induces a strong upregulation of both KLF4 and  $\mu$ -protocadherin (Fig. 1C). The same experiment was also performed on HT29 cells (Fig. 1D and E) confirming the results observed in CaCo2 cells.

### Modulation of KLF4 expression alters $\mu$ -protocadherin levels

To investigate whether KLF4 regulates directly  $\mu$ -protocadherin expression, we analyzed the effects of KLF4 silencing or overexpression in CaCo2 cells. Fig. 2A depicts a QRT-PCR showing KLF4,  $\mu$ -protocadherin, p21<sup>waf-1</sup> and CDX2 mRNA levels in KLF4-silenced CaCo2 cells, untreated or treated with 20 mmol/L 5-ASA. In particular, 5-ASA did not induce  $\mu$ -protocadherin expression in CaCo2 cells when KLF4 was silenced. p21<sup>waf-1</sup> and CDX2 are control genes induced by 5-ASA. Fig. 2B shows the protein levels of KLF4 and  $\mu$ -protocadherin in the same experimental setting. Again, 5-ASA failed to induce  $\mu$ -protocadherin expression in KLF4-silenced cells. To further investigate this relationship, we analyzed  $\mu$ -protocadherin expression in KLF4-overexpressing CaCo2 cells. By QRT-PCR analysis,  $\mu$ -protocadherin expression was found to increase in cells ectopically expressing KLF4, whereas p21<sup>waf-1</sup> and CDX2 levels did not change (Fig. 2C); these data suggest that of these three genes induced by 5-ASA only  $\mu$ -protocadherin expression might be directly regulated by KLF4.

### KLF4 directly regulates $\mu$ -protocadherin expression

To assess more directly whether KLF4 drives  $\mu$ -protocadherin expression, we inspected visually its

**Figure 1.**

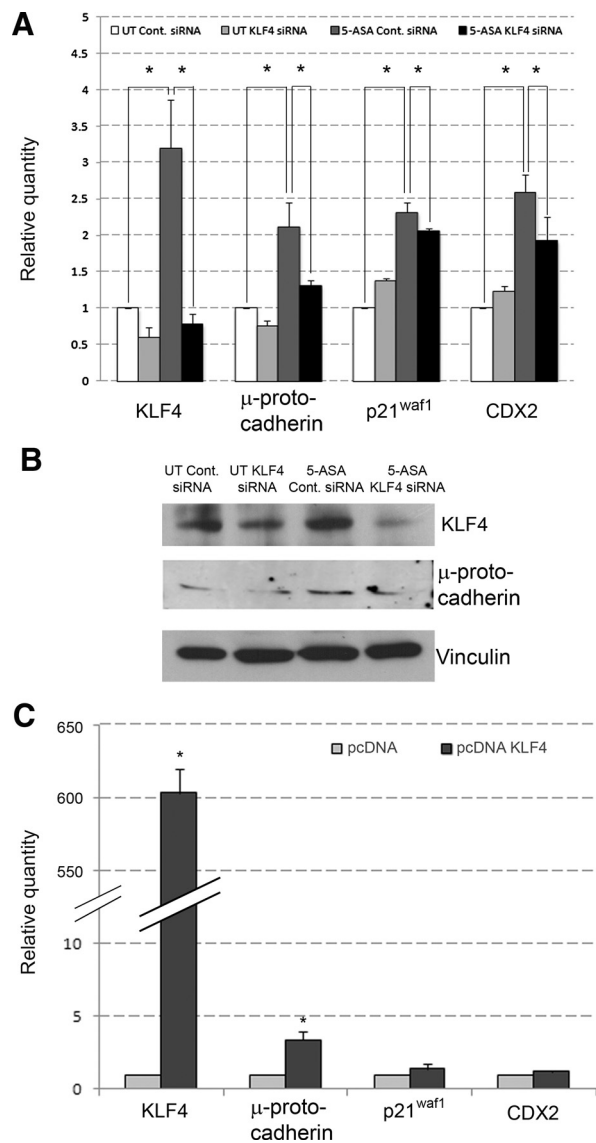
Effects of 5-ASA treatment on CaCo2 colorectal cancer cell line. **A**, 5-ASA causes an induction of KLF4 mRNA expression. Cells were untreated (UT) or treated with 20 mmol/L 5-ASA for 96 hours. mRNA expression was assessed by DNA microarray. The signal value is reported on y axis as an average of three independent experiments. Although these data are not statistically significant, they were intended as a starting point for further validation. **B**, 5-ASA induces upregulation of KLF4 transcripts. Cells were untreated (UT) or treated with the indicated doses of 5-ASA for 96 hours and then analyzed by QRT-PCR. Mean variations of mRNA expression levels from three independent experiments are reported in the y-axis as relative fold-change (relative quantity). Results are represented as mean  $\pm$  s.e.m. values and asterisks (\*) indicate statistically significant results ( $P < 0.05$ ). **C**, Western blot analysis of 5-ASA-treated CaCo2 cells shows an increased expression of KLF4 and  $\mu$ -protocadherin. Cells were untreated (UT) or incubated with the indicated doses of 5-ASA for 96 hours. Then, KLF4 Western blot analysis was performed on total protein extracts and normalized to Vinculin expression. Analyzed proteins are indicated on the right. **D** and **E**, Show the same experiments described in **B** and **C**, but performed in HT29 cells.

promoter for the presence of KLF4 consensus sequence and identified two putative binding sites (KRE1 and KRE2; Fig. 3A). The two sequences were separately cloned in the pT81Luc vector and their role in  $\mu$ -protocadherin expression was tested by luciferase reporter assays in HEK293T cells upon co-transfection with a plasmid driving KLF4 expression. Results of these assays suggest that only KRE2 may play a role in KLF4-induced  $\mu$ -protocadherin expression (Fig. 3B). Although not definitive, these assays strongly suggest that KLF4 drives directly  $\mu$ -protocadherin expression. Fig. 3C depicts a Western blot showing KLF4 protein

levels in HEK293T cells co-transfected with KLF4 and the luciferase reporter.

#### KLF4 inhibits Wnt/ $\beta$ -catenin transduction pathway by sequestering $\beta$ -catenin

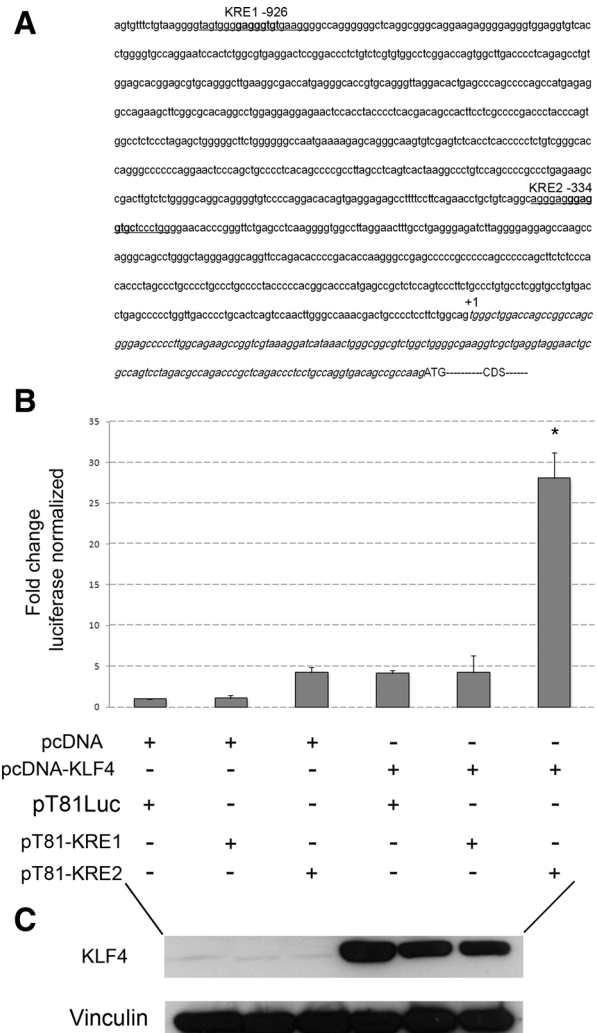
On the basis of these findings, it appears that 5-ASA induces KLF4 expression and that expression of KLF4 is required for 5-ASA-induced  $\mu$ -protocadherin expression.  $\mu$ -protocadherin inhibits Wnt signaling by sequestering  $\beta$ -catenin to the plasma membrane (6). Because KLF4 is capable of binding and sequestering  $\beta$ -catenin as well (10), we assessed if this interaction occurs



**Figure 2.** Effect of KLF4 modulation for 5-ASA-induced or basal  $\mu$ -protocadherin expression. **A**, CaCo2 cells were transfected with a scramble siRNA (Cont. siRNA) or a mix of anti-KLF4 siRNAs (KLF4 siRNA) and treated with 5-ASA, to induce  $\mu$ -protocadherin, CDX2 and p21<sup>waf-1</sup> expression, or cultured in absence of the compound (UT). Then, the effect on KLF4,  $\mu$ -protocadherin, CDX2 and p21<sup>waf-1</sup> mRNA levels was analyzed by QRT-PCR. Results are represented as mean  $\pm$  s.e.m. values of at least five independent experiments and asterisks (\*) indicate statistically significant results ( $P < 0.05$ ). **B**, Western blot analysis assessing the effect of KLF4 silencing on 5-ASA-induced  $\mu$ -protocadherin expression. **C**, CaCo2 cells were transfected with pcDNA KLF4 and its effect on the expression of  $\mu$ -protocadherin, CDX2 and p21<sup>waf-1</sup> was analyzed by QRT-PCR. Results are represented as in **A**.

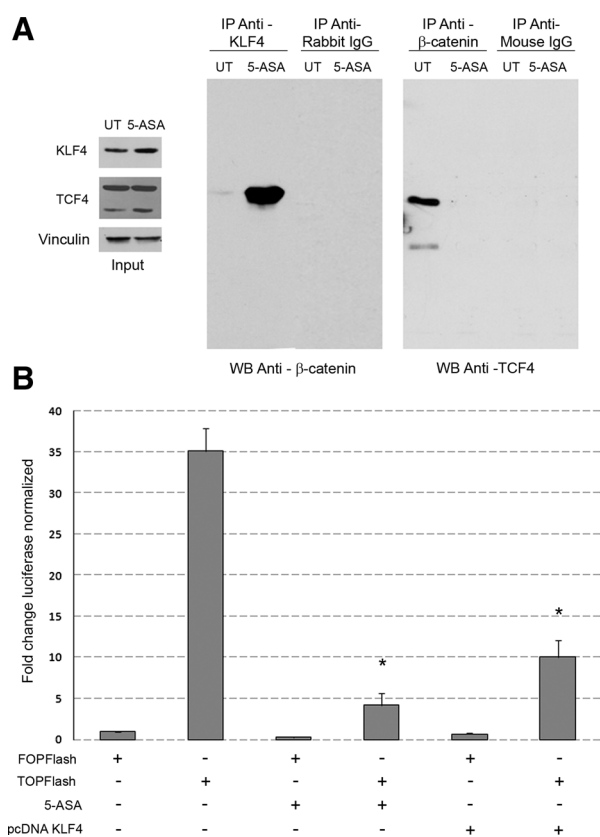
in 5-ASA-treated colorectal cancer cells. Thus, we immunoprecipitated KLF4 and  $\beta$ -catenin in CaCo2 cells treated with 5-ASA and performed a Western blot with anti- $\beta$ -catenin and anti-TCF4 antibodies. Results are shown in Fig. 4A. In particular, Fig. 4A (left) shows that

KLF4 expression is induced by 5-ASA treatment in the cell lysates used for the co-immunoprecipitation experiments; Fig. 4A (middle) shows that  $\beta$ -catenin co-immunoprecipitates with KLF4 only in cells treated with 5-ASA whereas TCF4 co-immunoprecipitates with  $\beta$ -catenin only in the absence of 5-ASA (Fig. 4A, right). This experiment suggests that, following 5-ASA treatment, besides inducing  $\mu$ -protocadherin expression,



**Figure 3.** Luciferase assays performed to validate KRE1- and KRE2-binding elements. **A**, 1 Kb proximal promoter region of  $\mu$ -protocadherin gene. Transcriptional start site and the translation start site (ATG) are indicated by +1 and by uppercase letters, respectively. The location of the KRE elements, positioned at -334 bp and at -926 bp from +1, are highlighted. **B**, Histograms describing the results of luciferase assays performed in HEK293T cells transfected with the pT81Luc reporter plasmids and pcDNA3.1 KLF4 expression vector. The x-axis: combination of transfected plasmids; y-axis: fold change of  $\beta$ -galactosidase normalized luciferase values. Bars represent the fold change of the luciferase activity  $\pm$  s.e.m. of at least three independent experiments and asterisks (\*) indicate statistically significant results ( $p < 0.05$ ). **C**, Western blot analysis following plasmids transfection shows an increase of KLF4 expression in presence of pcDNA KLF4 plasmid.

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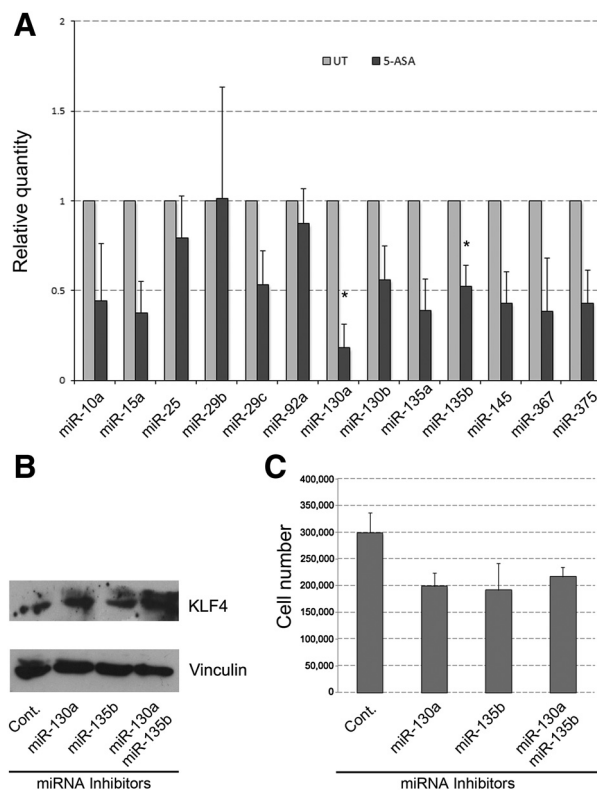


**Figure 4.** Analysis of KLF4  $\beta$ -catenin interaction by co-immunoprecipitation assay performed in 5-ASA-treated CaCo2 cells. **A**, Co-immunoprecipitation analysis showing the existence of a KLF4- $\beta$ -catenin protein complex in 5-ASA-treated (20 mmol/L) or -untreated (UT) CaCo2 cells. Western blot analysis of KLF4, TCF4, and Vinculin was performed on whole-cell extracts used as control (input, left side of the panel). Western blot analysis of  $\beta$ -catenin levels was performed on immunoprecipitated samples obtained with anti-KLF4 and normal rabbit IgG antibodies. TCF4 blotting was performed on extracts immunoprecipitated with anti- $\beta$ -catenin and normal mouse IgG antibodies (right side of the panel). IP, immunoprecipitation; WB, Western blot. Data are representative of three experiments and asterisks (\*) indicate statistically significant results ( $p < 0.05$ ). **B**, Histograms showing the results of luciferase assays performed in CaCo2 cells transfected with the TOPFlash or FOPFlash reporter plasmids in presence or absence of 5-ASA treatment or KLF4 overexpression. The *x*-axis, combination of transfected plasmids; *y*-axis, fold change of  $\beta$ -galactosidase normalized luciferase values. Bars represent fold changes of luciferase activity  $\pm$  s.e.m. of at least three independent experiments.

KLF4 is also capable of directly sequestering  $\beta$ -catenin. Moreover, this effect is accompanied by the release of TCF4 from  $\beta$ -catenin, which prevents its transcription activity. To further confirm these observations, we performed a reporter assay in CaCo2 cells using the TOP-flash system that allows to monitor the activity of the Wnt/ $\beta$ -catenin pathway. In particular, Fig. 4B shows that these cells display a strong basal activity of the Wnt/ $\beta$ -catenin pathway. This activity was strongly reduced by either 5-ASA treatment or KLF4 ectopic expression (Fig. 4B), confirming the role played by KLF4 in the regulation of the Wnt/ $\beta$ -catenin pathway.

### 5-ASA-induced KLF4 expression might depend on miR-130a and miR-135b downregulation

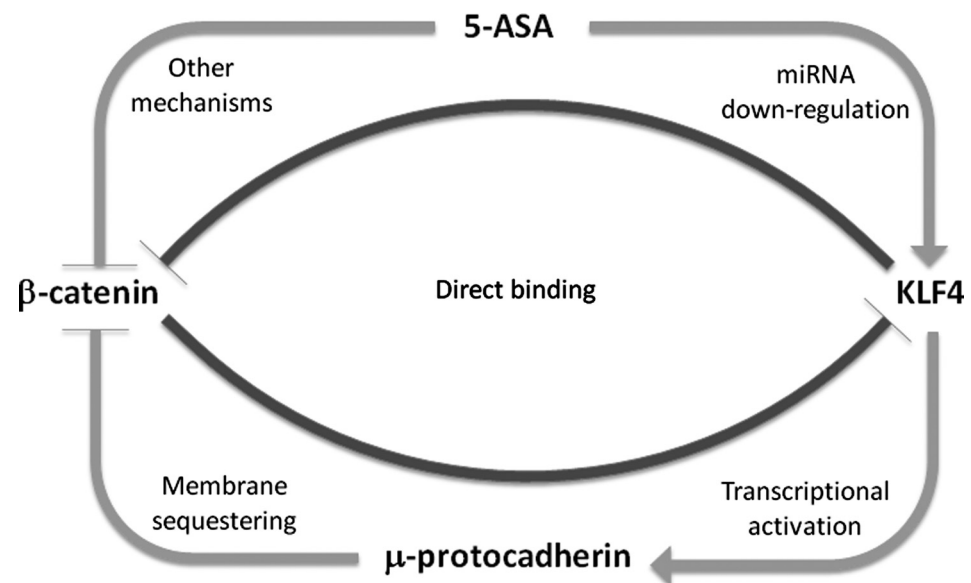
We performed a QRT-PCR analysis of 754 known microRNAs in CaCo2 cells treated with 5-ASA. Among the analyzed miRNAs, 13 (shown in Fig. 5A) were predicted by Targetscan software to directly target the KLF4 mRNA. Among these, only miR-130a and miR135b exhibited a statistically significant decrease in expression upon 5-ASA treatment of CaCo2 cells. Therefore, it is conceivable that 5-ASA-mediated KLF4 induction might be due to miR-130a and miR135b downregulation. To test this hypothesis, CaCo2 cells were transfected with specific miRNA inhibitors (anti-miR130a and anti-miR135b). Fig. 5B depicts a Western blot performed 48 hours post-transfection showing that inhibition of miR-130a and miR-135b determines an increase of KLF4 expression, thereby mimicking the effect of 5-ASA. Fig. 5C shows counts of silenced cells under the same experimental



**Figure 5.** **A**, Effects of 5-ASA treatment on miRNAs expression in the CaCo2 cell line. Histograms indicate the expression of miRNAs-targeting KLF4, predicted by Target Scan, analyzed by QRT-PCR. Results are represented as mean  $\pm$  s.e.m. values of three independent experiments and asterisks (\*) indicate statistically significant results ( $P < 0.05$ ). UT, untreated cells; 5-ASA, cells treated with 5-ASA. **B**, Western blot analysis of CaCo2 cells transfected with anti-miR-130a and anti-miR-135b miRNA inhibitors. Cells were lysed 48 hours post-transfection. **C**, Cell count performed on the same samples of **B**. Bars represent cell numbers  $\pm$  s.e.m. of three independent experiments.

**Figure 6.**

Diagrammatic representation of the molecular effects determined by 5-ASA on  $\beta$ -catenin activity in colorectal cancer cells. In this model, 5-ASA treatment leads to a miRNA-mediated upregulation of KLF4 which, in turn, induces  $\mu$ -protocadherin expression via enhanced transcription. Then, this protein sequesters  $\beta$ -catenin to the cell membrane resulting in the inhibition of its activity, also determined by 5-ASA through additional molecular mechanisms. The picture is completed by the reciprocal direct inhibition between KLF4 and  $\beta$ -catenin.



conditions. Although not statistically significant, the lower cell counts of samples transfected with miRNA inhibitors, compared with controls, is in accord with an increase of KLF4 expression.

## Discussion

Previous work suggested that treatment with 5-ASA might be chemopreventive for colorectal cancer (6, 19). In particular, treatment with 5-ASA induced  $\mu$ -protocadherin expression, promoting the sequestration of  $\beta$ -catenin to the plasma membrane and, consequently, the inactivation of Wnt/ $\beta$ -catenin signaling. In this study, we provide further insight on the chemopreventive activity of 5-ASA, suggesting the existence of a dual mechanism of  $\beta$ -catenin inactivation dependent on the transcription factor (TF) KLF4. This TF is not only known for its role in stem cells but also because its expression is often deregulated in cancer. For instance, its deletion in the intestinal epithelium results in an increase in the number and migration of epithelial cells and in the activation of genes of the Wnt pathway (20). Moreover, its expression is lost in colorectal cancer (21) and KLF4 is involved in methylation and "loss of heterozygosity" disease-related events (22).

In this study, we provide evidence that 5-ASA, besides inducing  $\mu$ -protocadherin, also activates KLF4 expression and that the latter is directly responsible for 5-ASA-dependent  $\mu$ -protocadherin expression. This conclusion is based on the finding that KLF4 silencing suppresses 5-ASA-mediated  $\mu$ -protocadherin expression and the results of luciferase reporter assays demonstrating the presence of a functional KLF4-binding site in the  $\mu$ -protocadherin promoter. As previously reported (10), KLF4 is also capable of directly binding and sequestering  $\beta$ -catenin in the nucleus.

Here we confirm by co-immunoprecipitation experiments that, upon 5-ASA treatment, KLF4 interacts with  $\beta$ -catenin preventing its binding to TCF4. On the basis of these findings, we suggest that 5-ASA inhibits the Wnt/ $\beta$ -catenin pathway by sequestering  $\beta$ -catenin to the plasma membrane ( $\mu$ -protocadherin mediated) and by inhibiting its transcriptional activity in the nucleus (KLF4 mediated). KLF4 plays a pivotal role in both mechanisms because it directly drives  $\mu$ -protocadherin expression.

To address potential mechanisms involved in 5-ASA regulation of KLF4 expression, we analyzed by QRT-PCR the levels of several micro-RNAs in CaCo2 cells treated with 5-ASA. We found that, among several others, miR-130a and miR-135b are strongly downregulated by 5-ASA treatment. Both micro-RNAs are involved in cancer. In particular, upregulation of miR-135b promotes chemoresistance (23) and cancer progression (24) in colorectal cancer, whereas miR-130a is associated with drug resistance and acts as an intermediate in the Wnt/ $\beta$ -catenin pathway (25). More importantly, both microRNAs were shown to downregulate KLF4 expression (26), supporting the idea that their decreased expression plays an important role in the 5-ASA-mediated increase in KLF4 expression. Altogether, these data further strengthen the notion that 5-ASA has a chemopreventive role in colorectal cancer providing a more complex picture of its mechanism of action, as shown in Fig. 6.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

Conception and design: S. Parenti, A. Grande

Development of methodology: S. Parenti, L. Montorsi, S. Fantini, F. Mammoli, C. Gemelli, C.G. Atene, L. Losi, C. Frassinetti

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**Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis):** S. Parenti, S. Fantini, E. Tagliafico  
**Writing, review, and/or revision of the manuscript:** S. Parenti, B. Calabretta, E. Tagliafico, T. Zanocco-Marani, A. Grande  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** C. Frassinetti  
**Study supervision:** E. Tagliafico, S. Ferrari, A. Grande

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# Cancer Prevention Research

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