Bidirectional KCNQ1:β-catenin interaction drives colorectal cancer cell differentiation.

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Bidirectional KCNQ1:β-catenin interaction drives colorectal cancer cell differentiation

Raphael Rapetti-Mauss a,b,1, Viviana Bustos a,1, Warren Thomas a, Jean Mc Bryan a, Harry Harvey a, Natalia Lajczak a, Stephen F. Madden a, Bernard Pellissier b, Franck Borgese b, Olivier Soriani b,c,2, and Brian J. Harvey a,2,3

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The K+ channel KCNQ1 has been proposed as a tumor suppressor in colorectal cancer (CRC). We investigated the molecular mechanisms regulating KCNQ1:β-catenin bidirectional interactions and their effects on CRC differentiation, proliferation, and invasion. Molecular and pharmacologic approaches were used to determine the influence of KCNQ1 expression on the Wnt/β-catenin signaling and epithelial-to-mesenchymal transition (EMT) in human CRC cell lines of varying stages of differentiation. The expression of KCNQ1 was lost with increasing mesenchymal phenotype in poorly differentiated CRC cell lines as a consequence of repression of the KCNQ1 promoter by β-catenin:T-cell factor (TCF)-4. In well-differentiated epithelial CRC cell lines, KCNQ1 was localized to the plasma membrane in a complex with β-catenin and E-cadherin. The colocalization of KCNQ1 with adherens junction proteins was lost with increasing EMT phenotype. ShRNA knock-down of KCNQ1 caused a relocalization of β-catenin from the plasma membrane and a loss of epithelial phenotype in CRC spheroids. Overexpression of KCNQ1 trapped β-catenin at the plasma membrane, induced a patent lumen in CRC spheroids, and slowed CRC cell invasion. The KCNQ1 ion channel inhibitor chromanol 293B caused membrane depolarization, redistribution of β-catenin into the cytosol, and a reduced transepithelial electrical resistance, and stimulated CRC cell proliferation. Analysis of human primary CRC tumor patient databases showed a positive correlation between KCNQ1:KCN3 channel complex expression and disease-free survival. We conclude that the KCNQ1 ion channel is a target gene and regulator of the Wnt/β-catenin pathway, and its repression leads to CRC cell proliferation, EMT, and tumorigenesis.

KCNQ1 | β-catenin | colon cancer | epithelial-mesenchymal transition | adherens junctions

The development of colorectal cancer (CRC) is determined by multiple factors including ion transport (1, 2). During the last 10 years, evidence for the role of K+ channels in the development and growth of tumors has greatly expanded. Voltage-gated K+ channels (Kv) are involved in the proliferation of many cell types, including intestinal cells. Although the recent literature clearly demonstrates that Kv channels are among the targets of interest in the fight against cancer (3–5), the specific role of each Kv channel in tumorigenesis and the molecular mechanisms involved are unknown. This is notably the case of the KCNQ1 K+ channel. The KCNQ1 gene has recently been identified as a tumor suppressor in mouse and human CRC tissues (6). KCNQ1 deficiency in mice caused rectal adenomatous hyperplasia and progression to adenocarcinoma. A loss of imprinting of KCNQ1 has been described in CRC (7). However, the functional and molecular events linking KCNQ1 and CRC progression remain unclear. One obvious pathway, which may interact with KCNQ1, is Wnt/β-catenin signaling, which plays a key role in driving early embryogenesis, as well as intestinal homeostasis and stem cell renewal in the intestinal mucosa epithelia (8). Deregulation of the β-catenin signaling axis is present in more than 80% of CRCS. This can lead to β-catenin accumulation in the cytosol, increased nuclear translocation of activated β-catenin, interactions with members of the T-cell factor (TCF) family, and stimulation of β-catenin-dependent gene expression, leading to increased cell proliferation and growth (9). In CRC cells, excessive nuclear accumulation of β-catenin was shown to increase the transcription of KCNQ1OT1 (10), a long noncoding RNA known to negatively regulate KCNQ1 expression. A possible link between β-catenin and ion channels in CRC has never been established. In this study, we demonstrate a bidirectional interaction between KCNQ1 and β-catenin regulating CRC cell differentiation processes. We also demonstrate that KCNQ1 is a target gene of β-catenin:TCF4, and that the expression, as well as the ion channel function, of KCNQ1 modulate epithelial phenotype. Moreover, KCNQ1 expression and its regulatory channel subunit KCNE3 (11) were correlated with better CRC patient survival.

Results

Loss of KCNQ1 Is Associated With Mesenchymal Phenotype in CRC Cells. In normal colonic epithelium, KCNQ1 drives Cl− secretion and generates the resting membrane potential (12, 13). Low expression of KCNQ1 has been correlated with CRC development in APC mouse models, but the function of the channels remains unknown (6). Using Western blotting, we compared the expression of KCNQ1 protein in six CRC cell lines of varying differentiation states and observed a high expression in

Significance

The K+ channel KCNQ1 has been proposed as a tumor suppressor in colorectal cancer (CRC), but nothing is known about its regulatory role in early disease stages. KCNQ1 is a target gene of Wnt/β-catenin, which is tonically activated in CRC. We demonstrate a bidirectional interaction between KCNQ1 and β-catenin as a key regulator of CRC cell differentiation, proliferation, and invasion. KCNQ1 stabilizes β-catenin at adherent junctions to maintain an epithelial phenotype. The β-catenin: T-cell factor (TCF)-4 transcriptional pathway directly represses KCNQ1 expression, and the loss of KCNQ1 was associated with an epithelial–mesenchymal transition. The KCNQ1:KCN3 ion channel complex expression in primary tumors was correlated with good survival outcome for patients with CRC. KCNQ1 is a potential early prognostic biomarker for CRC.


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The activation of β-catenin increased using two inhibitors of glycogen synthase kinase 3β expression, using a pharmacologic approach. We chose SW480 cells because of their high constitutive expression of β-catenin. Treatment with GSK3-IX to increase β-catenin activation resulted in enhanced recruitment of β-catenin and TCF4 proteins to the KCNQ1 promoter (Fig. 2 B and C). Furthermore, the luciferase assay showed that the KCNQ1 reporter transcriptional response was reduced in CHO cells treated with GSK3-IX compared with vehicle-treated control (Fig. 2D). To confirm the role of TCF4 in the control of KCNQ1 expression, DLD-1 and SW480 cells, which show low constitutive KCNQ1 expression, were transfected with a plasmid carrying a dominant negative mutant of TCF4 (hΔNTCF4), which cannot form a complex with β-catenin. This experiment revealed that the expression of KCNQ1 was restored in the presence of hΔNTCF4 in both cell lines (Fig. 2 E and F). Taken together, these data provide a molecular mechanism by which the activated Wnt/β-catenin pathway inhibits KCNQ1 protein expression via direct binding of a repressive β-catenin:TCF4 complex at the KCNQ1 promoter.

KCNQ1 Modulates β-Catenin Interactions at Adherens Junctions. In situ proximity ligation assay (PLA) revealed that KCNQ1 binds to both E-cadherin and β-catenin, which are markers of adherens junctions (AJ). In rat colonic crypts, KCNQ1 and β-catenin were observed by PLA to be in close association (Fig. 3 A and SI Appendix, Fig. S6). PLA dots were mainly observed at cell-to-cell contacts, and this was verified in HT29cl.19A cells, showing that KCNQ1 is a component of AJ in close contact (<40 nm) with β-catenin and E-cadherin (Fig. 3 B and SI Appendix, Fig. S6).

Activation of β-Catenin Causes Direct Transcriptional Repression of KCNQ1. We investigated the role of β-catenin in KCNQ1 expression, using a pharmacologic approach. β-catenin activity was increased using two inhibitors of glycogen synthase kinase 3β (GSK3β): AR-A014418 (AR-A 20 μM) or GSK3-inhibitor X (GSK3-X). The activation of β-catenin and its translocation into the nuclear fraction after inhibition of GSK3β was verified in HT29cl.19A cells (SI Appendix, Fig. S2). Activation of β-catenin reduced KCNQ1 total abundance in HT29cl.19A cells by 60% (Fig. 2A) and reduced the colocalization of KCNQ1 with β-catenin at the plasma membrane (SI Appendix, Fig. S3). Similar effects of pharmacologic agents were observed in HT29 cells (SI Appendix, Fig. S4). In addition to the pharmacologic approach, we also used a plasmid expressing a truncated form of β-catenin (β-catenin) to constitutively activate β-catenin. HT29cl.19A and HT29 cells transfected with activated β-catenin showed a loss of KCNQ1 and a redistribution of β-catenin from the plasma membrane into the cytosol (SI Appendix, Fig. S5). Chromatin immunoprecipitation (ChIP) and luciferase assays were performed to determine the effects of β-catenin activation on KCNQ1 transcription. We chose SW480 cells because of their high constitutive expression of β-catenin.
KCNQ1 Expression Suppresses Mesenchymal Features in CRC Spheroids. Knock-down of KCNQ1 induced a significant increase in mesenchymal markers (claudin-1, β-catenin, and N-cadherin), with a concomitant reduction in epithelial markers (ZO-1 and E-cadherin) (Fig. 4A). When grown on a 3D matrix, highly differentiated CRC cells such as HT29cl.19A underwent cytogenesis and generated spheroid structures. HT29cl.19A spheroids showed characteristic accumulation of F-actin at the apical membrane, β-catenin lateral staining, a patent central lumen, and well-defined adherens junctions (Fig. 4B). Knock-down of KCNQ1 significantly impaired the ability of HT29cl.19A cells to form spheroids, and the frequency of lumen formation was reduced from 80% to 20% (Fig. 4B).

We examined the effects of overexpression of KCNQ1 in an intermediate EMT cell line HCT116 with low endogenous expression levels of KCNQ1. KCNQ1 construct expression in HCT116 cells led to an increase in epithelial markers (ZO-1 and E-cadherin) and a decrease in mesenchymal markers (β-catenin and N-cadherin) (Fig. 4A). The overexpression of KCNQ1 in HCT116 cells restored the ability of this moderately differentiated CRC cell line to form spheroid structures, and the frequency of lumen formation was increased from 19% to 62% (Fig. 4B). Collectively, these results demonstrate that KCNQ1 is required to maintain a well-differentiated epithelial phenotype.

**KCNQ1 Ion Channel Function and Membrane Voltage Modulate CRC Phenotype and β-Catenin Subcellular Distribution.** The function of KCNQ1 as an ion channel is essential to maintain a hyperpolarized negative membrane voltage in colorectal cancer (11, 15). We observed that KCNQ1 overexpression in HCT116 cells induced a redistribution of β-catenin and KCNQ1 at the plasma membrane (Fig. 4A) and membrane hyperpolarization (Fig. 4B). The membrane hyperpolarization was exclusively generated by KCNQ1 channels, as the change in membrane potential was inhibited by the KCNQ1 selective channel blocker chromanol 293B (Fig. 4B). The inhibition of outward K+ current flow through KCNQ1 channels will produce a membrane depolarization. The effects of membrane depolarization on KCNQ1 and β-catenin

Furthermore, silencing of KCNQ1 in HT29cl.19A cells using ShRNA resulted in a significant disruption of E-cadherin:β-catenin interactions (Fig. 3C). These observations indicate the importance of KCNQ1 in maintaining AJ stability and tight junction formation. This was further demonstrated by the reduction in transepithelial electrical resistance (TEER) in HT29cl.19A cells in which KCNQ1 expression had been silenced (Fig. 3D). Silencing of KCNQ1 also resulted in a redistribution of β-catenin from the plasma membrane to a more diffuse cytosolic location (SI Appendix, Fig. S7A). Consistent with this observation, KCNQ1 silencing resulted in altered phosphorylation of β-catenin at specific residues that determine its subcellular distribution and stability. The level of p-S33 phosphorylation that induces the degradation of cytosolic β-catenin (15, 16) was lower in the KCNQ1 knockdown cell lines (Fig. 3E). In contrast, phosphorylation at residues Y654 and S675, which favor the stabilization of cytosolic β-catenin and its translocation into the nucleus, was significantly increased in KCNQ1-silenced cells (Fig. 3E). These results lend strong support for the conclusion that KCNQ1 is a pivotal regulator of β-catenin stability and subcellular distribution. As a corollary, the data indicate that KCNQ1 suppresses the Wnt/β-catenin signaling pathway. This was indeed shown to be the case where silencing KCNQ1 expression activated signaling intermediates of the Wnt/β-catenin pathway, increasing the expression of p-AKT, p-GSK-3β, cyclin D-1, cJun, and Met (SI Appendix, Fig. S7B and C).

**KCNQ1 Expression Suppresses Mesenchymal Features in CRC Spheroids.** Knock-down of KCNQ1 induced a significant increase in mesenchymal markers (claudin-1, β-catenin, and N-cadherin), with an
cells were subjected to a 3D Matrigel invasion assay.

cellular distribution was produced by two methods: collapsing the electrochemical gradient for K+ by exposure to high extracellular K+ and inhibiting outward K+ currents with a specific KCNQ1 ion channel blocker. Membrane depolarization triggered by exposing the cells to high external K+ (predicted membrane potential from the Nernst equation: +2 mV), caused a rapid (within 5 min) redistribution of KCNQ1 and β-catenin from the plasma membrane into the cytosol (Fig. 6C). Blocking KCNQ1 channels with chromanol 293B (C293B) produced a similar cytosolic redistribution of KCNQ1 and β-catenin (Fig. 6D). Transepithelial electrical resistance is determined mainly by cell–cell adhesion properties of tight junctions. Two selective blockers (C293B and HMR) of KCNQ1 ion channels produced a decrease in ZO-1 expression and a reduced TEER over the course of 10 d of cell culture (SI Appendix, Fig. S8). Taken together, these data demonstrate that the function of KCNQ1 as an ion channel is essential for KCNQ1 and β-catenin interaction and the formation of a polarized epithelium.

KCNQ1 Suppresses Proliferation and Invasion of CRC Cells. Given the modulation of β-catenin by KCNQ1, we hypothesized that KCNQ1 expression could modulate CRC cell proliferation. Treatment of HT29cl.19A cells with GSK3-ix to activate β-catenin and decrease KCNQ1 expression resulted in an increase in cell proliferation (SI Appendix, Fig. S9A). Inhibition of the KCNQ1 channel with C293B also resulted in an increase in proliferation (SI Appendix, Fig. S9B). Conversely, increasing KCNQ1 activity by transfection of DLD-1 cells with hΔN-TCF4 resulted in a decrease in cell proliferation (SI Appendix, Fig. S9C). Treatment of hΔN-TCF4 transfected DLD-1 cells with C293B to block KCNQ1 channels rescued the proliferative capacity (SI Appendix, Fig. S9D). Overexpression of KCNQ1 in HCT-116 cells resulted in a decreased proliferation (SI Appendix, Fig. S9E). To evaluate the role of KCNQ1 in cell invasion, cells were subjected to a 3D Matrigel invasion assay. HCT116 cells overexpressing the KCNQ1 construct showed a lower rate of invasion compared with wild-type cells of low KCNQ1 expression (SI Appendix, Fig. S10). These data demonstrate that KCNQ1 channels act to repress proliferation and invasion of CRC cells.

The KCNQ1 Channel Complex Is Associated with CRC Patient Survival. The KCNQ1 functional channel complex in colon is composed of the KCNQ1 pore-forming unit and a KCNE3 regulatory subunit. In a cohort of 355 human primary colon tumor samples, Kaplan Meier and log rank test analysis demonstrated that CRC relapse-free survival was significantly higher for patients with high KCNQ1 (Fig. 7A) and high KCNE3 mRNA expression (Fig. 7B). TCF4, which we have shown to suppress the KCNQ1 promoter, was inversely correlated with KCNQ1 expression in these patients (SI Appendix, Fig. S11). High TCF4 expression was significantly correlated with low patient survival probability (Fig. 7C). Although KCNQ1 was correlated with patient survival, we found no significant correlation between KCNQ1 expression and tumor stage (SI Appendix, Table S1).

Discussion

Our study demonstrates that KCNQ1 is a target gene for the Wnt/β-catenin pathway. We also uncovered a function of KCNQ1 in regulating β-catenin activity at the plasma membrane. The loss of KCNQ1 promotes the disruption of cell–cell contact, contributing to EMT, cell proliferation, and invasion. The function of KCNQ1 as an ion channel appears to be involved in these processes. We describe the molecular mechanisms of KCNQ1:β-catenin bidirectional interactions and a signaling pathway for the tumor suppressor activity of KCNQ1 in CRC. A number of studies have linked K+ channel deregulation to carcinogenesis (4, 5), but the underlying molecular mechanisms have remained largely unknown. In this study, we demonstrate a positive correlation between high KCNQ1 expression and CRC cell epithelial phenotype and patient survival in primary stage
The characterization of signaling pathways regulating ion channel expression in cancers is an open question, and very few cell-cell contact. This role for KCNQ1 is supported by our findings that KCNQ1 silencing induced a loss of sphereoid organization and lumen formation in CRC 3D cultures. We propose a model in which KCNQ1 is essential for epithelium integrity by stabilizing AJ proteins, repressing EMT by retaining β-catenin at the plasma membrane, thus limiting β-catenin:TCF4 transcriptional activation of proliferative genes. In addition, our results suggest this function depends, in part, on the regulation of the membrane potential by the channel. Electrophysiological analyses in many cancer cell types have shown that membrane depolarization favors cell proliferation (26). We demonstrated that KCNQ1 maintains a hyperpolarized Vm in CRC cells, and experimental maneuvers designed to collapse the outward K⁺ currents through KCNQ1 channels disrupted the normal KCNQ1:β-catenin interaction at the plasma membrane and produced a more mesenchymal phenotype. We conclude that the functional endpoint of the bidirectional KCNQ1:β-catenin interaction is the regulation of proliferative and invasive potency of CRC cells.

The loss of KCNQ1 expression is associated with poor survival in patients with CRC liver metastases (6), and a recent study has shown that low KCNQ1 mRNA expression correlates with poor disease-free survival in stage II colon cancers (27). In our analysis of human primary tumors from patients with stage I to IV disease, we observed that high expression of KCNQ1 was significantly associated with a better patient survival outcome. TCF4 expression showed an inverse correlation with survival outcome in our patient cohort that is consistent with its role in repressing KCNQ1. Intriguingly, we also found a direct correlation between survival outcome and high expression of KCNE3, the regulatory subunit of the KCNQ1 ion channel in human colon. We also found the KCNQ1 expression, although being significantly associated with survival, is independent of tumor stage. This increases the attractiveness of KCNQ1:KCNE3 as a predictive marker and a therapeutic target in CRC.

Taken together, our study demonstrates a bidirectional interaction between KCNQ1 and β-catenin, which controls both their expression and subcellular localization to modulate CRC cell phenotype, proliferation, and invasion.

**Materials and Methods**

**CRC Cell Culture and Spheroid Formation.** HEK293T and CHO cell lines, as well as the CRC cell lines HT29, HT29cl.19A, SW480, SW620, DLD-1, and HCT116, were maintained in Dulbecco’s modified Eagle’s or RPMI medium with 10% FBS. 3D-culture CRC spheroids were developed in Growth Factor Reduced Matrigel (BD Biosciences), as further described in SI Appendix, Materials and Methods. No human material requiring consent was used in our experiments.

**Animals and Isolation of Colonic Crypts.** Male Sprague-Dawley rats (200–250 g) were obtained from Janvier Labs and maintained at the laboratory animal house during a short period of 24 h (local ethics committee approval was
received from the University of Nice Sophia-Antipolis). Animals were killed by lethal intraperitoneal injection of pentobarbital. The colon was removed by dissection, and colonic crypts were isolated as previously described in ref. 12.

Plasmid Transfection and ShRNA Transduction. Two stable KCNQ1 shRNA knock-down cell lines were engineered using two different DNA sequences (SI Appendix, Materials and Methods) The double-stranded DNA sequences were inserted in the mammalian expression vector pSuperRT Puro, a derivative of the pPRIG series. Transduction experiments were performed as previously described (28, 29). Cells were transiently transfected using lipofectamine 2000 (Invitrogen), according to the manufacturer’s recommendation. DLD-1 and SW480 cells were transfected with 2 μg ΔhN-TCF4 plasmid (30). HT29cl.19A and HT29 were transfected in suspension with 2 μg j-cataR87 plasmid (31). KCNQ1 transduction of HTC116 cells was performed as described previously (32). More details are given in SI Appendix, Materials and Methods.

Transepithelial Electrical Resistance. HT29cl.19A cells were grown on semi-permeable supports (Merck Millipore), and TEER was measured in triplicate with an EVOM electrometer (WPI), using triplicates for each measurement every 2 for 10 d. More details are given in SI Appendix, Materials and Methods.

Immunoblotting and Immunofluorescence. Whole-cell lysates were prepared and subjected to 8% SDS/PAGE before proteins being transferred onto a nitrocellulose membrane. Membranes were probed with the primary antibody overnight at 4 °C. Reactive proteins were developed with HRP- and Methods

Chromatin Immunoprecipitation. ChIP was carried out using a ChIP-IT High Sensitivity Kit (Active Motif), as previously described (33). More details are provided in SI Appendix, Materials and Methods.

In situ Proximity Ligation Assay. The DuoLink PLA (Olink Bioscience) was used to detect interactions among KCNQ1, β-catenin, and E-cadherin. HT29cl.19A cells were seeded on poly-L-lysine (40 μg/mL)-coated microscope slides. CRC cells and isolated rat colonic crypts were fixed with paraformaldehyde 4% and permeabilized with triton x100 (0.05%). Cells were immunolabeled with primary antibodies anti KCNQ1, Kv7.1 (1:100) and anti β-catenin (1:100), or anti E-cadherin (1:100) for 1 h at 37 °C. The secondary antibodies with attached PLA probes were supplied in the DuoLink kit. CELLA images were captured using an inverted Zeiss Axios Observer Z1 microscope (Zeiss). PLA dots per cells were quantified using Image J software.

Luciferase Reporter Assay. CHO cells were plated at 2 × 10^6 cells/well in 96-well plates. One day after plating, the cells were washed once with PBS and transfected with either LightSwitch GoClones KCNQ1 reporter (Active Motif), LightSwitch GAPDH Promoter Control (positive control; Active Motif), or LightSwitch Random Promoter Control 1 (negative control) (Active Motif), using FuGENE HD transfection reagent (Promega Corporation). After 24 h, transfected cells were treated with 40 nM GSK3α-iX (Merck Millipore) for another 24 h. Luciferase activities were measured using the LightSwitch Luciferase Assay Kit and LightSwitch Assay Substrate optimized for use with RenSP luciferase, using one-step reagent addition (Active Motif).

Statistical Analysis. Mann–Whitney or Kruskal-Wallis nonparametric test was applied to compare significance between two or more groups, as appropriate. The frequency of lumen formation in HT29cl.19A and HCT116 spheroids was analyzed using Fisher’s exact test. Values of n number of experiments are given as mean ± SEM. A P value <0.05 was considered statistically significant.

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Figure S1. KCNQ1 and β-catenin co-localization. (A) Van Steensel's analyses of KCNQ1 and β-catenin staining co-localization in HT29cl.19A cells, n= 15 from 6 independent experiments and (B) in isolated rat colonic crypts, n= 20 from 5 different rats. Pearson's coefficient is given by the (cross-correlation function) CCF value corresponding to x =0.
Figure S2. GSK3-iX increases the expression of activated nuclear β-catenin. (A) Western blot and densitometry analysis confirmed that treatment of HT29cl.19A cells with the GSK3-inhibitor X (GSK3-iX) resulted in an increased expression of nuclear activated β-catenin (ABC). Lamin A/C was used as loading control. (B) Western blot analysis confirmed that GSK3-iX treatment resulted in a decreased expression of cytosolic activated β-catenin (ABC). β-Actin was used as a loading control (n=3, ***P<0.001; ****P<0.0001).
Figure S3. GSK-3β inhibitors decrease the co-localization of KCNQ1 and β-catenin. (A) Confocal immunofluorescence imaging of the subcellular distribution of KCNQ1 (green) and β-catenin (red) following activation of β-catenin with AR-A014418 (AR-A 20μM) or GSK3-inhibitor X (GSK3-iX 5μM) in HT29cl.19A cells. Merged images indicate co-localization (yellow) between KCNQ1 and β-catenin staining. Scale bars 10 μm. n=3. (B) Van Steensel's analyses of KCNQ1 and β-catenin staining co-localization in HT29cl.19A under control condition (black line), treated with AR-A (red line) or treated with GSK3-iX (blue line) n=10 from 4 independent experiments. Pearson's coefficient is given by the CCF value corresponding to x=0.
**Figure S4. GSK-3β inhibitor effects on KCNQ1 and β-catenin subcellular localization.** (A) Confocal immunofluorescence imaging of the subcellular distribution of KCNQ1 (green) and β-catenin (red) following activation of β-catenin with two inhibitors of GSK-3 β; AR-A (20µM) and GSK3-iX (5µM) in HT29 cells. The merged images indicate co-localization (yellow) of KCNQ1 with β-catenin under control conditions. (B) Western Blot and densitometry analysis of KCNQ1 expression in HT29 cells following modulation of β-catenin activity. Cells were treated with; AR-A (20µM) or GSK3-iX (5µM) to stabilize activated β-catenin (n= 4; ***P< 0.001).
Figure S5. Constitutive activation of β-catenin decreases KCNQ1 expression and re-localizes β-catenin in CRC cells. Subcellular distribution of KCNQ1 (green) and β-catenin (red) following transfection (24h) of HT29 (A) and HT29cl.19A (B) cells with a constitutively activated mutant of β-catenin (β-catenin∆N87) or an empty vector (EV). Images are representative of 3 independent experiments.

Figure S6. Controls for Proximity Ligation Assay. (A) PLA between KCNQ1 and β-catenin in HEK-T cells which lack KCNQ1 expression. (B) PLA in HT29cl.19A without primary antibodies were used as negative controls. Nuclear DAPI staining is in blue (n=3). Scale bars 15µm.
Figure S7. Silencing of KCNQ1 induces AKT phosphorylation and increased Wnt pathway activity. (A) Confocal immunofluorescence images showing subcellular localization of β-catenin (red) in HT29cl.19A ShKCNQ1 cells line (ShQ1-1 and ShQ1-2) or in the control HT29cl.19A ShRD cell line (n=3). Orthogonal views are shown on the side of merge images. Scale bars 20µm. (B) Western blot and densitometry analysis of KCNQ1 silencing (ShQ1-1 and ShQ1-2) effects on AKT (S473) and GSK3-β (S9) phosphorylation in HT29cl.19A cells. GAPDH was used as a loading control, n=4 *** p<0.001 (C) Western blot and densitometry analysis of Wnt pathway target proteins expression (Cyclin D1, C-Jun and Met) in ShRD or KCNQ1 knock-down HT29cl.19A cells (ShQ1-1 and ShQ1-2). Tubulin was used as a loading control, (n=4 ** p<0.01, ***p<0.001).
Figure S8. KCNQ1 regulation of transepithelial electrical resistance.
Transepithelial electrical resistance (TEER) in HT29cl.19A epithelial monolayers, treated with KCNQ1 channel blockers chromanol 293B or HMR 1556 (n=6; ***P≤ 0.001). In insert, the expression of ZO-1 protein is shown.

Figure S9. KCNQ1 channel regulation of CRC cell proliferation (A) Proliferation of HT29.cl19A cells was increased following β-Catenin activation 24h post-treatment with GSK3-iX (40nM and 5μM). Mean ± SEM, n=6. *P<0.05, ***P=0.003. (B) Blocking the KCNQ1 channel in HT29.cl19A cells, with the
specific inhibitor C293B (10µM or 100µM) for 24h increased cell proliferation (n=3, **P<0.05). (C) Enhanced KCNQ1 expression in DLD-1 cells transfected with h∆N-TCF4 caused a decrease in cell proliferation measured 48h post-transfection compared with pcDNA3.1 (Empty Vector), (n=6, ****P<0.0001). (D) Ion channel block of KCNQ1 in h∆N-TCF4 transfected DLD-1 cells enhanced cell proliferation. Cell proliferation of control (Empty Vector pcDNA3.1) and transfected DLD-1 cells was measured 24h post-treatment with C293B (10 µM or 100 µM), (n=3, ****P<0.001). (E) MTS assay showing the effect of treatment with C293B (10µM) for 24h or ectopic expression of KCNQ1 construct (HCT116-Q1) on HCT 116 proliferation (n=4, *P<0.05).

Figure S10. Over-expression of KCNQ1 channel reduces invasion of HCT116 cells. Representative images and quantification of Matrigel® invasion
assays. Cells were seeded in growth-free matrigel drops and incubated at 37°C for 60h (bar 30µm, n=4; *p<0.05).

Figure S11. Correlation between KCNQ1 and TCF4 expression in CRC patients. KCNQ1 mRNA expression inversely correlates with TCF4 in a cohort of 355 colon tumour samples (data sets GSE14333 and GSE17538). Tumors were ordered from high to low expression of KCNQ1 (correlation coefficient, R=-0.308, p=3x10^-9).
Table S1. Comparison between KCNQ1 expression levels and tumour stage. Colon cancer data sets GSE14333 and GSE17538 were used to test for correlation between KCNQ1 expression and tumour stage. Two probes (204487_s_at, 211217_s_at) for the KCNQ1 gene were used on the Affymetrix Human Genome U133 Plus 2.0 microarray platform. No correlation was found between probe expression level and tumour stage. See Supplemental Methods for more details.

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<td>-0.04</td>
<td>0.56</td>
</tr>
<tr>
<td>GSE17538</td>
<td>204487_s_at</td>
<td>-0.03</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>211217_s_at</td>
<td>0.02</td>
<td>0.82</td>
</tr>
</tbody>
</table>

SI Materials and Methods

Antibodies

The following antibodies were purchased. Cell Signaling Technology: Phospho-β-Catenin (Ser33/37), Phospho-β-Catenin (Ser675), Lamin A/C, Claudin-1, ZO-1. Sigma: KCNQ1 (SAB2108683), β–Actin (A5441), AKT, p-AKT (S473), GSK-3β, p-SK-3β (S9), α-Tubulin (DM1A). BD Transduction laboratories: β- catenin (610153), E-Cadherin, N-Cadherin. Merck Millipore: Anti-Active β- catenin (ABC) clone 8E7 (05-665). Thermo Scientific: c-Myc Epitope Tag Antibody (9E11). Santa Cruz Biotechnology: E-Cadherin (sc-8426), N-Cadherin (sc-393933), Pol II (sc-899), TCF4 (sc- 8631), Cyclin D1, C-Jun, MET, normal rabbit IgG (sc-2027). Abcam: Phospho-β-Catenin (Y654). Molecular Probe: Phalloidin (Alexa 568).
Spheroid formation

HT29cl.19A or HCT116 cells were trypsinized to a single cell suspension of 5×10^3 cells/ml in 0.5% FBS completed with 2% GFR Matrigel. Cells in GFR Matrigel were seeded in 8-well coverglass chambers (Nalge Nunc) precoated with 100% GFR Matrigel (for 30min at 37°C). Cells were fed every 2 days and grown for 10 days.

ShRNA transduction

The DNA sequences of KCNQ1 ShRNA are as follow: 5'-GTA GAT CTT CCA GGT GGA GTT TTT TGG AAA-3' (shQ1-1) and 5'-GAT CCC GTC TTT GCC ATC TTC TTC TTC GCT CGA GCA AAG AAG GAG ATG GCA AAG ATT TTT TGG AAA-3' (shQ1-2).

Plasmid transduction

cDNA coding sequence of KCNQ1 was subcloned into the modified mammalian expression vector pPRIG-eGFP where eGFP was exchanged for puromycin resistance sequence in order to generate pPriPu vector. Retroviruses were produced transfecting HEK 293T cells with pPriPu/KCNQ1, pCMV-VSVG, pCMV-gag-pol plasmids, using the classic calcium phosphate transfection technique. Replication-defective retroviruses were recovered in the culture medium between 48h post-transfection. This retroviral supernatant was filtered through sterile 0.45 µm filters, then added directly to HTC 116 cells in the presence of 4 µg/ml polybrene to enhance retroviral transduction efficiency. 3 days later, puromycin was added in fresh medium to start selection of transduced cells.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) in SW480 cells was carried out using ChIP-IT® High Sensitivity Kit (Active Motif, USA) as previously described (33). The name and sequences of the primers, cycles and annealing temperature for each pair are listed in Table S2.
Table S2. Specific primer sequences for ChIP assay

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Primer Sequence</th>
<th>Annealing (ºC)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (F)</td>
<td>5'-CCAGCAAGAGCACAAGAGGAAGAG-3'</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>GAPDH (R)</td>
<td>5'-CAAGGGGCTCTACATGGCAACTGTG-3'</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>KCNQ1 (F/R)</td>
<td>NM_181798.1 (-)17Kb: GPH1002055(-)17A</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>KCNQ1 (F/R)</td>
<td>NM_000218.2 (-)01Kb: GPH1002054(-)01A</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

Trans-epithelial electrical resistance

In pharmacological experiments, HT29cl.19A cells were grown on semi-permeable supports (Merck Millipore, 4.2cm²) and the TEER measured in triplicate every second day for 10 days in control conditions or treated with two different inhibitors of the KCNQ1 channel, Chromanol 293B (Tocris) or HMR 1556 (Tocris). Following the 10th day of TEER measurements, the level of ZO-1 expression was quantified by Western blot analysis. In KCNQ1 shRNA knockdown experiments, the TEER was measured as above in HT29cl.19A Sh RD and HT29cl.19A ShQ1-1 and ShQ1-2 transduced cells grown on semi-permeable supports (Merck Millipore, 0.75cm²). Expression levels of KCNQ1 were determined by Western blot after 10 days of culture.

Cell proliferation and growth assays

Cell proliferation in vitro was measured by MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corporation). HT29, HT29cl.19A (1x10⁴ cells/well), DLD-1 and HCT116 (5x10³ cells/well) cells were plated in 96-well plates in triplicate. After 24h of culture, HT29 and HT29cl.19A cells were treated for 24h with 40nM and 5µM of GSK3-iX (Merck Millipore) to stabilize the activated β-catenin or with 10µM and 100 µM of Chromanol 293B (Tocris) to block KCNQ1 selectively. Cell proliferation was measured 24h post-treatment with GSK3-iX. Cell proliferation of DLD-1 cells was measured 48h post-transfection with pcDNA3.1 (Empty Vector) or pcDNA3.1/hΔN-TCF4. The absorbance was determined using a MultisKan EX Microplate ELISA Reader.
(Thermo Electron, UK) at a wavelength of 490 nm. The growth rates of cells were calculated using the following formula: growth rate = (1 - average value of untreated control well/average value of experimental well) × 100%. DLD-1 cells were transfected with pcDNA3.1 (Empty Vector) or pcDNA3.1/hΔN-TCF4 and 48h post transfection were seeded at a density of 1×10^4 in 24-well plates and incubated for 5 days. Cells were then trypsinized and viability was measured manually by haemocytometer counting using trypan blue (Sigma) exclusion assay.

**Reverse transcription and quantitative real-time PCR**

Total RNA was extracted using Total RNeasy Mini Kit (Qiagen). A total of 1µg of RNA was reverse-transcribed into cDNA using ImProm-II™ Reverse Transcription System (Promega Corporation). The cDNA was subsequently used as a template for 40 cycles of PCR with primers specific for human KCNQ1 (or GAPDH which was used as a normalization control). SYBR®Green (Thermo Scientific) qPCR analyses were performed in triplicate on 7500 Fast Real-Time PCR System (Applied Biosystems). Total RNA of 3 different sets of cells was used to analyze gene expression. KCNQ1 mRNA expression levels were calculated relative to HT29cl.19A cell line. The sequences of the primers, cycles and annealing temperature for each pair are listed in Table S3.

**Table S3. Gene-specific primer sequences for qPCR.**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Primer Sequence</th>
<th>Annealing</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (F)</td>
<td>5'-GTCATCATCTGCCCCCTCTGCT-3'</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td>GAPDH (R)</td>
<td>5'-CGACGCCTGCTTCACCACCTTCT-3'</td>
<td>58</td>
<td>40</td>
</tr>
<tr>
<td>KCNQ1 (F)</td>
<td>5'-GCTGAGAAAGATCGCGTTAAG-3'</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>KCNQ1 (R)</td>
<td>5'-AGAAACAGGAGGCCGATGCT-3'</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>
**Matrigel drops invasion assay**

HCT 116 control cells and HCT116 expressing KCNQ1 (4X10^5 cells) were included in 40% growth-factor-reduced Matrigel drops and incubated in 10% FBS at 37°C, 5% CO₂ in an incubator associated to a time laps microscope (Zeiss). Images were recorded every hour for 60 hours.

**Kaplan-Meier analysis**

Kaplan-Meier analysis of relapse-free patient survival in 286 primary colon tumors was carried out using the R2 bioinformatics web tool on previously published data sets GSE14333 and GSE17538 (R2: Genomics analysis and visualization platform [http://r2.amc.nl](http://r2.amc.nl)). Median gene expression was used to stratify patients into high or low expression for each gene. P-values were calculated based on a log rank test.

Utilising the previously published colon cancer data sets GSE14333 and GSE17538 used to perform the survival analysis, we attempted to establish if there was a significant correlation between KCNQ1 expression and tumor stage. These studies used the Affymetrix Human Genome U133 Plus 2.0 microarray to measure the colon cancer transcriptome. There are two probes (204487_s_at, 211217_s_at) for the KCNQ1 gene on this microarray platform. Gene expression values were called using the GeneChip (GC) robust multichip average method (1) and the data was quantile normalised using the Bioconductor package, affy ([http://www.bioconductor.org/](http://www.bioconductor.org/)). For each probe, we calculated Pearson’s correlation coefficient between probe expression level and tumour stage.

**Van Steensel's cross correlation function**

The cross-correlation function (CCF) of a dual labeling image was calculated by shifting the red image over a distance Δx pixels in the x-direction with respect to the green image, with −25 ≤Δx ≤25. A negative value of Δx indicates that the red image was shifted to the left, a positive value indicates a shift to the right. For each value of Δx Pearson’s correlation coefficient was calculated with a maximal value for Δx=0.
**Electrophysiology**

Electrophysiological recordings in HCT116 or HCT116 expressing KCNQ1 cells were performed at room temperature in the whole cell configuration of the patch clamp technique. Current-clamp experiments were carried out to record the resting membrane potential. The bath solution was: (mM) 5 KCl, 140 NaCl, 1 MgCl₂, 1 CaCl₂, and 10 Hepes (pH adjusted to 7.4 with HCl, 285 mosm/l). Soft glass patch electrodes (Brand, Wertheim, Germany) were made on a horizontal pipette puller (P-97, Sutter Instrument Co., Novato, CA) to achieve a final resistance ranging from 3 to 5 MΩ. The pipette solution was (mM) 130 KCl, 1 MgCl₂, 1 CaCl₂, 10 EGTA, 10 Hepes, 2 ATP (pH adjusted to 7.2 with KOH, 290 mosm/l). Electrical signals were amplified with an Axopatch 200B amplifier (Molecular Device, Foster City, CA) and acquired with a DIGIDATA 1440 interface and pCLAMP 10.2 software (Axon Instruments).

Bath application of C293B (10µM) was used to selectively block KCNQ1.

For immunofluorescence imaging using high K⁺ stimulation, we incubated for 5 minutes HT29cl.19A cells grown in coverslips with control Krebs solution (in mM): 124 NaCl, 1.5 CaCl₂, 4.7 KCl, 1.2 MgSO₄, 25 NaHCO₃, 0.8 KH₂PO₄, 10 Glucose. In experiments in which high K⁺ buffer was used, a sufficient volume of K-Gluconate was added to the standard buffer to obtain the final desired concentration of K⁺. This 100% high K⁺-buffer had the same composition as the Krebs buffer, except that 135 mM K-Gluconate was substituted for 124 mM NaCl/4.7 mM KCl. Both solutions were bubbled with a mixture of 5% CO₂ and 95% O₂, giving a pH of 7.4.

**Reference:**