Supporting Information

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SI Materials and Methods

Antibodies. Antibodies against the different claudins were purchased from Zymed (Invitrogen). Monoclonal antibody against Ki67 was from Neomarkers, and anti-activated caspase 3 and phosphohistone H3 antibodies were from Cell Signaling (Ozyme). Anti-Symplekin antibodies were from Becton Dickinson or from Progen. Antibodies against actin were from Sigma.

Preparation of Nuclear Extracts. Nuclear extracts were prepared using the Nuclei EZ Prep nuclei isolation Kit (Sigma) according to the manufacturer's instructions.

Western Blot. HT29-Cl.16E cells were lysed in 1× radioimmunoprecipitation assay (RIPA) buffer as previously described (1). Twenty micrograms of total protein lysate were loaded per lane of 7–10% SDS/PAGE gels, and Western blotting was performed as described in ref. 2. Antibody concentrations were anti-symplekin (1/5000), anti-actin (1/4000), anti-claudin-2 and -4 (1/500), anti-claudin-7 (1/1000), and anti-claudin-15 (1/250). Optimal exposure times for membranes were used, and protein expression was quantified using National Institutes of Health Image 1.62, adjusted for background noise and protein loading.

Immunofluorescence. Cells were grown on glass coverslips in 12-well plates until ready for processing, then were washed two times in PBS, fixed on ice for 30 min in 95% ethanol, and postfixed for 1 min with acetone. Alternatively, cells were fixed in 3% paraformaldehyde for 15 min at room temperature and permeabilized in PBS/0.2% Triton X-100 for 10 min. For zonula occludens 1-associated nucleic acid-binding protein (ZONAB)/claudin-2 staining, cells were incubated in actin stabilization buffer and fixed in ice-cold methanol for 7 min at −20 °C and then in ice-cold acetone for 30 s at room temperature. Nonspecific sites were blocked using PBS, 0.5% BSA, 10 mM glycine or PBS, 5% BSA, 0.3% gelatin for 30 min. Primary antibodies were diluted in blocking buffer and incubated overnight at 4 °C. Secondary antibodies used were goat anti-mouse IgG-Alexa488, donkey anti-rabbit-Alexa594 (Molecular Probes/InVitrogen), or Cy3-coupled goat anti-rabbit IgG (Chemicon/Millipore). Samples were mounted in Mowiol (Aldrich). Confocal images were obtained using a Biorad MRC 1024 confocal setup (Optiphot2 microscope, with a Plan Apo 20×/0.75 lens; Nikon) and were mounted as figures using Adobe Photoshop.

SiRNA Transfections. The SMARTpool siRNA mixture against ZONAB/decorin-binding protein A and siRNA against human claudin-2 (5′-CUCUUUACUUGGGCAUUAU-3′) and symplekin (5′-GGCCUAUGAAACUCUGCAUTT-3′) were from Dharmacon (Perbio Science). HT29-Cl.16E cells were seeded into six-well plates at 2×10^5 cells/well and transfected the following day with 120 pmol siRNA using EXGEN 500 (Euromedex). Cells were harvested 72–96 h later, and total RNA or RIPA protein lysates were prepared, respectively. For high-confluency experiments, 10 μL of polyethylenimine (Exgen, Fermentas) was mixed with 4 μg of human symplekin cDNA (3) (or pCDNA-3 as control) or with 500 nM claudin-2, symplekin, or β-galactosidase (β-gal) siRNA, in a final volume of 100 μL of 150 mM NaCl. After 30 min at room temperature, the mix was added to 10^6 HT29Cl.16E cells in suspension, and cells were plated immediately in a six-well plate. At the specified time, cells were lysed, and RNA was extracted. Cell numbers and nucleotide and polyethylenimine quantities were halved when cell were seeded in 12-well plates for transepithelial resistance (TER) and cell polarity analysis.

RT- qPCR. Total RNA was prepared with the RNeasy kit (Qiagen). We used 2.5 μg of total RNA to prepare cDNA with M-MLV reverse transcriptase (Invitrogen). Amplification was conducted in a LightCycler (Roche Diagnostics) using the FastStart DNA Master SYBR Green I PCR kit. *GAPDH*, a common housekeeping gene, was used as an internal control. Primer sequences were forward: GAGAAGGCTGGGGCTCAT, reverse: TGCTGATGATCTTGAGGCTG for *GAPHD*; forward: TGAGTTCCTGCAGCCTCTG, reverse: CTCCACGGGGGTGTAGATG for *SYMPK*; and forward: GAGGGATTAGAGGTGTTCAAGG, reverse: AGGGACTGCTCCCTTGTCTT for *CLDN2*.

Measurement of Transepithelial Resistance and Dilution Potential. Cells were grown to confluency on a polyester filter membrane (Transwell 0.4-mm pore size, Costar). TER of confluent cell monolayers was measured every day using a Millicell-Electrical Resistance System from Millipore connected to a dual Ag-AgCl electrode. TER values are expressed in Ω/cm^2. Dilution potentials were measured as described in ref. 4 . Briefly, cells were grown on filter membranes and exposed to different media on their basolateral [buffer B: 120 mM NaCl, 10 mM Hepes (pH 7.4), 5 mM KCl, 10 mM NaHCO3, 1.2 mM CaCl2, and 1 mM $MgSO_4$] and apical [buffer A: 60 mM NaCl, 10 mM Hepes (pH 7.40, 5 mM KCl, 10 mM NaHCO3, 1.2 mM CaCl2, 1 mM $MgSO_4$] sides Iso-osmolarity was maintained by the addition of 145 mM mannitol to buffer A. All transepithelial voltages are reported as V1-V2, where the V1 electrode is in the apical compartment and V2 is in the basal compartment.

1. Hollande F, Lee DJ, Choquet A, Roche S, Baldwin GS (2003) Adherens junctions and tight junctions are regulated via different pathways by progastrin in epithelial cells. *J Cell Sci* 116(Pt 7):1187–1197.
2. Darido C, et al. (2008) Defective claudin-7 regulation by Tcf-4 and Sox-9 disrupts the polarity and increases the tumorigenicity of colorectal cancer cells. *Cancer Res* 68: 4258–426.
3. Hofmann I, Schnölzer M, Kaufmann I, Franke WW (2002) Symplekin, a constitutive protein of karyo- and cytoplasmic particles involved in mRNA biogenesis in *Xenopus laevis* oocytes. *Mol Biol Cell* 13:1665–1676.
4. Colegio OR, Van Itallie CM, McCrea HJ, Rahner C, Anderson JM (2002) Claudins create charge-selective channels in the paracellular pathway between epithelial cells. *Am J Physiol Cell Physiol* 283:C142–C147.

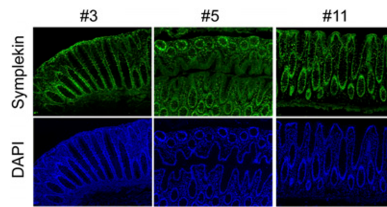


Fig. S1. Immunofluorescence (IF) detection of symplekin in three human colon epithelial samples. Similar staining was obtained from eight colorectal cancer (CRC) samples, and results from other samples within this patient population are shown in ref.1. Patient identification numbers correspond to CRC samples shown in Fig. 1*B*.

1. Buchert M, et al. (2009) The symplekin/ZONAB complex inhibits intestinal cell differentiation by the repression of AML1/Runx1. *Gastroenterology*, 137:156–164, 164 e151–e153.

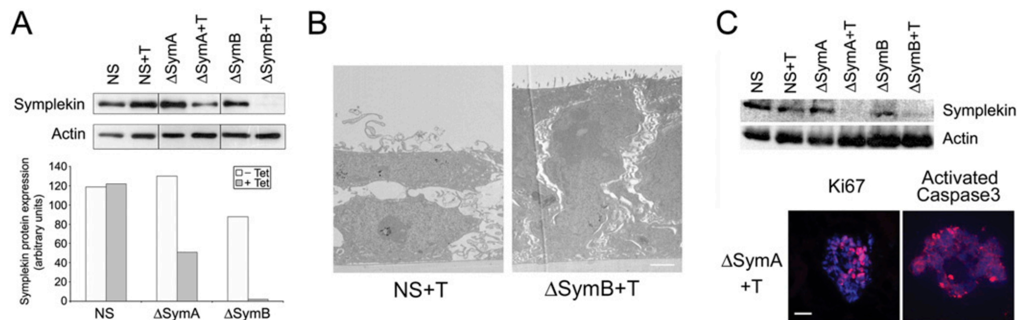


Fig. S2. (*A*) Representative photomicrograph of symplekin Western immunoblotting in HT29-Cl.16E cells transfected with a control nonspecific shRNA construct (NS) or with shRNAs specifically directed against different regions of the symplekin mRNA (ΔSymA, ΔSymB) and treated or not treated with tetracycline (−tet, white bars; +tet, gray bars), as described in *Materials and Methods*. (*B*) Ultra-structural analysis was performed on cells grown on Transwell filters for 10 days post-confluence in the presence of tet, using transmission electron microscopy. (Scale bar, 3 m.) Similar results were obtained for clone DSymA. (*C*) (*Top*) Control, ΔSymA, and ΔSymB cells were grown in Matrigel for 14 days with (+Tet) or without tetracycline and lysed. Symplekin down-regulation after tetracycline treatment was confirmed using Western immunoblotting. Actin was used as a loading control. (*Bottom*) Proliferation and apoptosis were analyzed in cryosections of ΔSymA cells grown as in Fig. 3, using Ki67 or activated caspase 3 staining, respectively. Nuclei are stained with DAPI. (Scale bars, 10 μm.)

A

Claudin isoform	Cycle threshold (Ct) in HT29NS + Tet cells	Fold induction in Dsym + Tet cells (DsymA and DSymB)
CLDN1	27.08 +/- 2.01	1.23 +/- 0.69
CLDN2	26.47 +/- 1.85	0.37 +/- 0.05
CLDN3	20.43 +/- 0.65	1.10 +/- 0.24
CLDN4	22.35 +/- 1.15	1.07 +/- 0.33
CLDN5	>32	NA
CLDN6	>35	NA
CLDN7	23.84 +/- 2.40	1.59 +/- 0.75
CLDN8	>32	NA
CLDN9	29.75 +/- 0.80	0.77 +/- 0.22
CLDN10 transcript 1	>35	NA
CLDN10 transcript 2	>35	NA
CLDN11	>35	NA
CLDN12	>35	NA
CLDN14 transcript 1	>32	NA
CLDN14 transcript 2	29.04 +/- 2.72	1.10 +/- 1.13
CLDN15	28.44 +/- 0.90	4.28 +/- 0.94
CLDN16	>35	
CLDN17	31.40 +/- 0.31	1.48 +/- 0.37
CLDN18 transcript 1	>35	NA
CLDN18 transcript 2	>35	NA
CLDN19 transcript 1	>35	NA
CLDN19 transcript 2	>35	NA
CLDN20	>35	NA
CLDN22	29.94 +/- 0.72	0.87 +/- 0.73
CLDN23	>35	NA

B

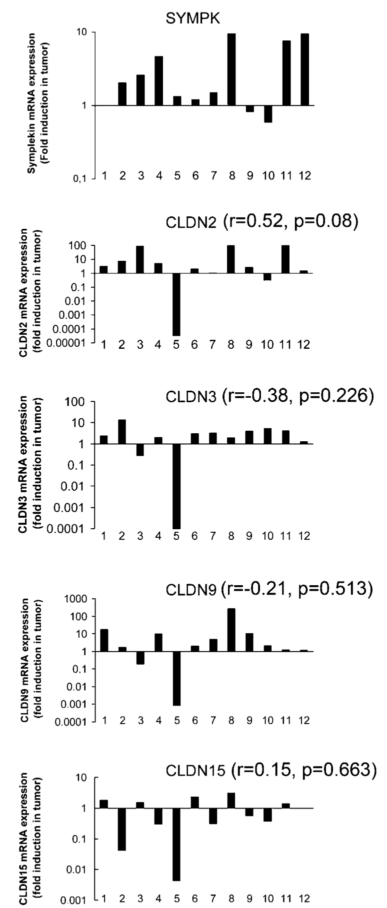


Fig. S3. Expression of claudin isoforms in correlation with symplekin levels in HT-29Cl.16E cells and human CRC biopsies. (*A*) (*Middle*) Expression of mRNAs for claudin isoforms was quantified using RT-qPCR in control HT-29Cl.16E NS cells. Values represent the cycle threshold for each isoform. (*Right)* mRNAs for claudin isoforms were quantified in uninduced and induced HT-29/Δsym cells. Results represent the fold-increase of each mRNA upon tetracycline-induced symplekin down-regulation. Values are mean ± SEM of levels in DSymA and DSymB clones. (*B*) mRNA expression for symplekin and claudin-2, -3, -9, and -15 in tumor samples from 12 patients with CRC, relative to the levels in their respective matching healthy epithelium, which were normalized to 1 for each patient (indicated by the horizontal axis). The histogram for symplekin is a different representation of the results shown in Fig. 1*A*, where corresponding tumor staging is provided. The degree of statistical correlation between symplekin expression and that of individual claudins across the patient population is provided for each claudin (r = Spearman's correlation coefficient for nonparametric samples).

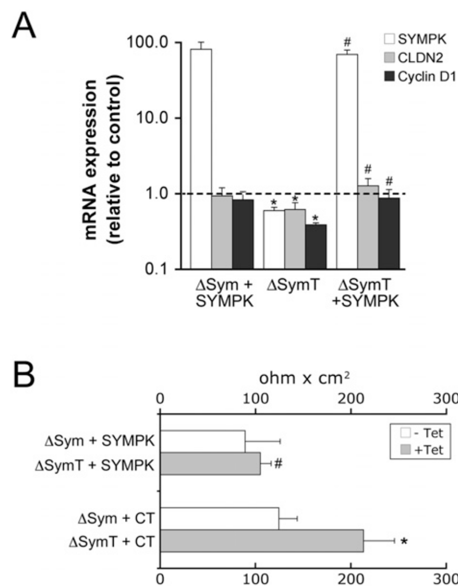


Fig. S4. Overexpression of full-length human symplekin rescues the phenotype induced by symplekin down-regulation on gene expression and TER. (*A*) Expression of symplekin (SYMPK), claudin-2 (CLDN2), and cyclin D1 mRNAs was quantified using RT-qPCR in ΔSym cells, induced with or without tetracycline and transfected with the full-length human symplekin construct (+ SYMPK) or with pcDNA3. Results are expressed relative to the expression level in pcDNA3-transfected HT29-Cl.16E ΔSym without tetracycline, represented by the dashed line. $P < 0.05$ compared with control cells (*) or with tetracycline-treated ΔSym cells transfected with pcDNA3 (#); Student *t* test; $n = 3$. (*B*) TER measurement on ΔSym cells, induced with or without tetracycline, transfected in suspension with the full-length human symplekin construct (+ SYMPK) or with pcDNA3 (+CT), and then seeded at high density on Transwell filters and grown for 4 days after transfection. $P < 0.05$ compared with ΔSym + CT cells (*) or with ΔSymT + CT cells (#), $n = 2$).

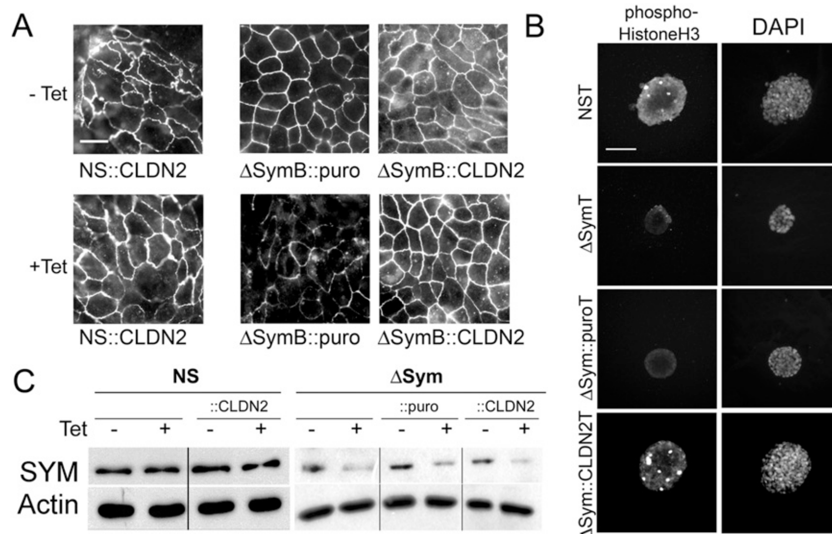


Fig. S5. Restoration of claudin-2 expression reverses the effects of symplekin down-regulation in HT29-Cl.16E ΔSym cells. (*A*) IF detection of claudin-2 (CLDN2) in NS and ΔSym cell lines stably transduced with a retroviral construct expressing claudin-2 (::CLDN2) or a control empty vector (::puro), and grown in the presence (+ tet) or absence (– tet) of tetracycline. (Scale bar, 20 μm.) (*B*) IF detection of phosphohistone H3 in suspension spheroids grown from control (NS) and from symplekin-depleted cells (ΔSym), transduced or not with a control (ΔSym::puro) or a claudin-2-expressing construct (ΔSym::CLDN2), as shown in Fig. 6. Nuclei are stained with DAPI. (Scale bar, 50 μm.) (*C*) Western immunoblotting demonstrating that symplekin down-regulation induced upon tetracycline treatment is not reversed after transduction of ΔSym cells with a retroviral construct expressing claudin-2 (::CLDN2) or a control empty vector (::puro). Actin was used as a loading control.

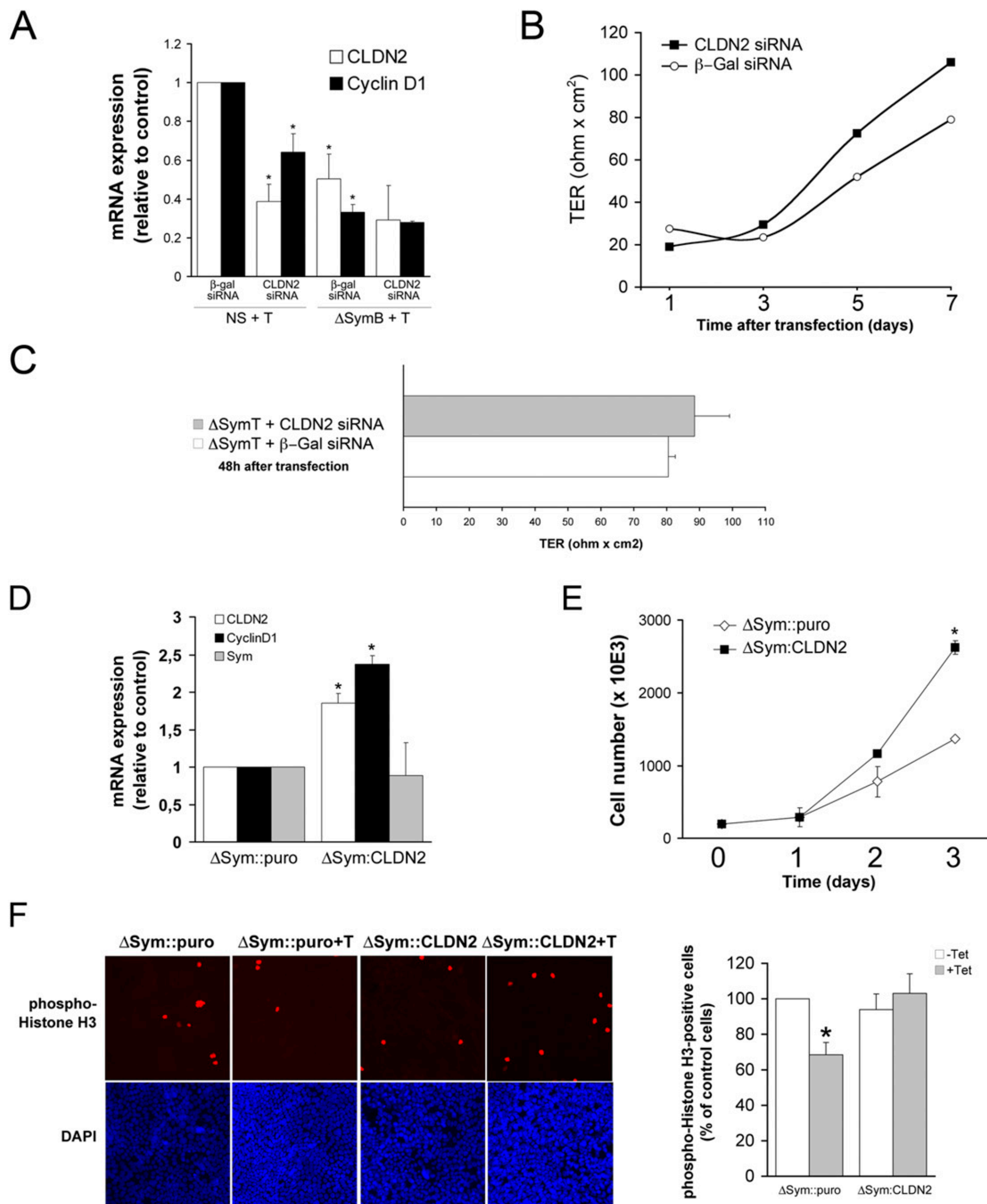
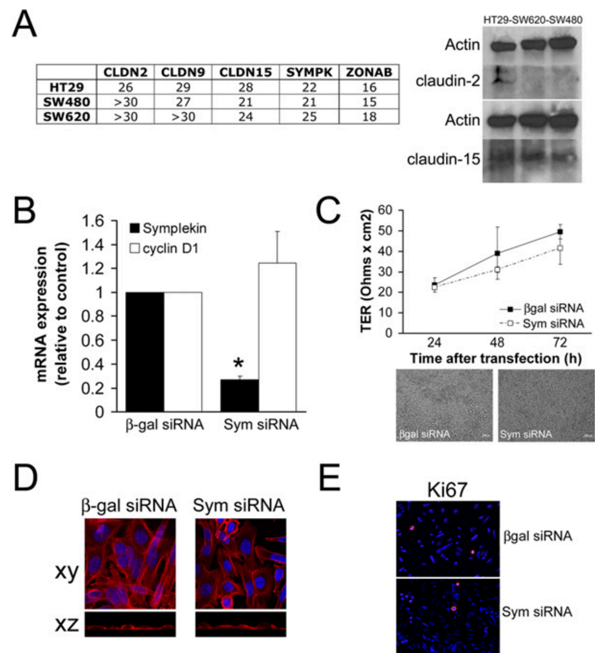


Fig. S6. Experimental modulation of claudin-2 expression affects gene expression, paracellular ion transport, and cell proliferation in HT29-Cl.16E CRC cells. (*A*) Expression of claudin-2 (CLDN2) and cyclin D1 mRNAs was quantified using RT-qPCR in NS+T and ΔSymB+T cells, transfected with β-gal or *CLDN2*-selective siRNA. Results are expressed relative to the expression level of each gene in β-gal siRNA-transfected NS + T cells. (*, $P < 0.05$ compared with control cells; $n = 2$). (*B*) Time-course measurements of TER on NS cells, transfected in suspension with β-gal or *CLDN2*-selective siRNA, then seeded at high density on Transwell filters and grown for up to 7 days after transfection. Results summarize one of two similar experiments. (*C*) TER measurement in ΔSymT cells, induced or not with tetracycline, transfected in suspension with β-gal or *CLDN2*-selective siRNA, then seeded at high density on Transwell filters and grown for 4 days after transfection. ($n = 3$). (*D*) Expression of claudin-2, cyclin D1, and symplekin mRNA was quantified using RT-qPCR in ΔSym::puro and ΔSym::CLDN2 cells without tetracycline. Results are expressed relative to the expression level of each gene in ΔSym::puro cells. (*, $P < 0.05$; Student *t* test; $n = 3$). (*E*) ΔSym::puro and ΔSym::CLDN2 cells (2×10^5/well) were seeded and grown without tetracycline, and the number of cells in each well was counted after 1, 2, and 3 days in culture. (*, $P <$ 0.05 compared with ΔSym::puro cells, $n = 2$). (*F*) (*Left*) IF detection of phosphohistone H3 in ΔSym::puro and ΔSym::CLDN2 cells grown for 14 days after reaching confluence, with or without tetracycline. (*Right*) The histogram summarizes the quantification of phosphohistone H3-positive cells in each population. Results are expressed as a percentage of positive cells in ΔSym::puro cells without tetracycline (*, $P < 0.05$; Student *t* test; $n = 3$).



	CLDN2	CLDN9	CLDN15	SYMPK	ZONAB
HT29	26	29	28	22	16
SW480	>30	27	21	21	15
SW620	>30	>30	24	25	18

Fig. S7. siRNA-mediated symplekin down-regulation does not affect cyclin D1 expression, cell polarity, or proliferation in CRC cells lacking claudin-2. (*A*) (*Left*) Expression of mRNAs for claudin-2, -9, and -15, symplekin, and ZONAB was quantified using RT-qPCR in HT-29Cl.16E, SW480, and SW620 CRC cells. Values represent the cycle threshold for each mRNA. (*Right*) Expression of claudin-2 and claudin-15 was analyzed in HT-29, SW620, and SW480 cells using Western immunoblotting. Results show one of three similar experiments. (*B*) Expression of symplekin and cyclin D1 mRNAs was quantified using RT-qPCR in SW480 cells, 72 h after transfection with β-gal or symplekin-selective siRNA. Results are expressed relative to the expression level of each gene in SW480 cells transfected with β-gal siRNA. (*, $P < 0.05$; Student *t* test; $n = 3$). (*C*) (*Upper*) Time-course measurements of TER on SW480 cells, transfected in suspension with β-gal or symplekin-selective siRNA, and then seeded at high density on Transwell filters and grown for up to 3 days after transfection ($n = 3$). (*Lower*) Bright field images showing that cells used for time course TER measurements were at a similar degree of confluence. (*D*) xy- (*Upper*) and xz- (*Lower*) confocal sections were collected from TRITC-phalloidin–stained SW480 cells transfected with β-gal or symplekin-selective siRNA and grown for 3 days after transfection. (Scale bar, 50 μm.) (*E*) IF detection of Ki67 in SW480 cells, transfected in suspension with β-gal or symplekin-selective siRNA, seeded at high density, and grown for 3 days after transfection.

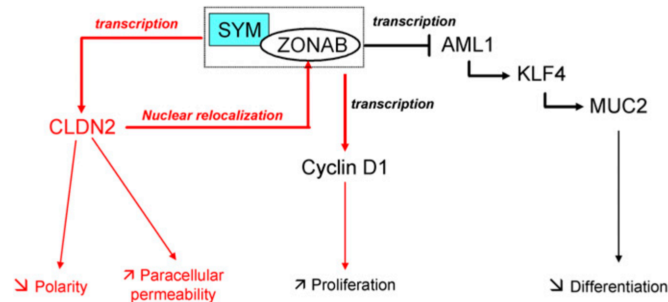


Fig. S8. Schematic representation of the role played by the symplekin/ZONAB complex in the regulation of proliferation, differentiation, polarity, and paracellular permeability in CRC cells. Red arrows and text correspond to results obtained in the present study. The transcriptional regulation of cyclin D1 by ZONAB was described previously in refs.1 and 2.

1. Sourisseau T, et al. (2006) Regulation of PCNA and cyclin D1 expression and epithelial morphogenesis by the ZO-1-regulated transcription factor ZONAB/DbpA. *Mol Cell Biol* 26:2387–2398.
2. Kavanagh E, et al. (2006) Functional interaction between the ZO-1-interacting transcription factor ZONAB/DbpA and the RNA processing factor symplekin. *J Cell Sci* 119:5098–5105.

Table S1. Primers for claudin, symplekin, and GAPDH qPCR

	Forward	Reverse
CLDN-1	CCC TAT GAC CCC AGT CAA TG	ACC TCC CAG AAG GCA GAG A
CLDN-2	GAGGGATTAGAGGTGTTCAAGG	AGGGACTGCTCCCTTGTCTT
CLDN-3	AACCTGCATGGACTGTGAAA	GGTCAAGTATTGGCGGTCAC
CLDN-4	CGGGATCCA CCACCATGG GGC TAC AGG TAATGG G	CCA CCA GCG GAT TGT AGA AG
CLDN-5	TCCTGTCTGAAGGCCAGAG	CCATCACACTGGGTGTAGCA
CLDN-6	TCTGTTTCCTCCGTCCTGATGCGGGGGTCTACATAGCTG	GCGGGGGTCTACATAGCTG
CLDN-7	aaaatgtacgactcggtgctc	cacttcatgcccatcgtg
CLDN-8	ATTCCCTGCTGGCTCTTTCT	GAAGCAGCACACATCAGTCC
CLDN-9	GAGACGTGGCTGTCCTCAG	GGTGCTGCTGCTCTTACCA
CLDN-10 TR1	gccgaccgcattttactatc	gcgcaaatatggaaccaaag
CLDN-10 TR2	ggacttcccctccatgct	gcgcaaatatggaaccaaag
CLDN-11	CCCGGTGTGGCTAAGTACAG	CAACAAGGGCGCAGAGAG
CLDN-12	CTGTTTTGGAACTGTCAGGTA	TTCCACACAGGAAGGAAAGG
CLDN-14 TR1	tcttgcacatgggagctg	atgagcatgtgcacttccag
CLDN-14 TR2	ggccgtgtcctacctgaa	actggtagatgcctgtgctgt
CLDN-15	CTGGGAGTTCCCGTCCAT	AGCAAGAGGCCGAGGAAG
CLDN-16	TGCTGGAGCCACGTTACTAAT	CACATACACATCAACAGCATACCA
CLDN-17	gtggggactcttgccaca	gctgccaacaaaagctgata
CLDN-18 TR1	aggccctatttcaccatcct	gacgatgcctacgatcatca
CLDN-18 TR2	cgagagagctctggcttcac	gacgatgcctacgatcatca
CLDN-19 TR1	agcagcccacagccctat	gcgggcaatttaacaactg
CLDN-19 TR2	aagctctacgactcgctgct	ccaactacgctgaggaccat
CLDN-20	CAGTGTCTGCTTGCTCTTCAA	CAGTAGAGAACCAAATAACAAAGC
CLDN-22	actggggtgtgtgggtagat	ggcagtgtgctccacaga
CLDN-23	ttgcatcaatataattattgggtttt	agtttgcatggcaaggagtt
SYMPK	TGAGTTCCTGCAGCCTGTG	CTCCACGGGGGTGTAGATG
GAPDH	GAGAAGGTGGGGCTCAT	TGCTGATGATCTTCAGGCTG