The Symplekin/ZONAB Complex Inhibits Intestinal Cell Differentiation by the Repression of AML1/Runx1

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BACKGROUND & AIMS: Symplekin is a ubiquitously expressed protein involved in RNA polyadenylation and transcriptional regulation that localizes at tight junctions in epithelial cells. The association between symplekin and the Y-box transcription factor ZONAB activates proliferation in intestinal and kidney cells. We analyzed symplekin expression in human colonic crypts and investigated its function in differentiation. **METHODS:** Expression of differentiation markers and transcription factors was assessed in HT29-Cl.16E cells that expressed inducible symplekin short hairpin RNA or were transfected with ZONAB small interfering RNAs. Intestines of AML1$^{\Delta/\Delta}$ mice were stained with alcian blue and analyzed for expression of AML1/Runx1, GAPDH, KLF-4, and Muc-2. Mobility shift and chromatin immunoprecipitation were used to detect AML1 and ZONAB/DbpA binding to promoter regions of the Krüppel-like factor 4 (*KLF4*) and acute myeloid leukemia-1 (*AML1*) genes, respectively. **RESULTS:** The gradient of nuclear symplekin expression decreased from the proliferative toward the differentiated compartment of colonic crypts; symplekin down-regulation promoted the differentiation of HT29-Cl.16E colorectal carcinoma cells into goblet cells. Down-regulation of symplekin or ZONAB/Dbpa induced de novo expression of the transcription factor AML1/Runx1, thereby increasing the expression of KLF4 and promoting goblet cell differentiation. Furthermore, increased AML1 expression was required for the induction of goblet cell differentiation after symplekin down-regulation. KLF4 expression and goblet cell numbers were reduced in the intestines of AML1$^{\Delta/\Delta}$ mice, confirming the role of AML1 as a promoter of intestinal differentiation in vivo. **CONCLUSIONS: Symplekin cooperates with ZONAB to negatively regulate intestinal goblet cell differentiation, acting by repression of AML1 and KLF4.**

The intestinal epithelium is a highly organized tissue in which the single epithelial sheet contains all cell types, from stem cells located at the bottom of the Lieberkühn crypts to terminally differentiated cells shedding into the lumen. Homeostasis of this permanently renewing organ can only be maintained because of a tight balance between proliferation, differentiation, migration, and apoptosis.[1] A number of proteins involved in cell–cell contacts have been implicated in the maintenance of this equilibrium, acting either as the source (eg, Notch[2]) or as downstream effectors of specific signaling pathways (eg, ephrins/Eph Receptors, E-cadherin/β-catenin).[1,2] Proteins localized in the submembrane plaque cell–cell junctions can also shuttle toward the cell nucleus, where they modulate nuclear events by their interaction with a variety of transcriptional regulators, as shown in the case of β-catenin,[1] p120 catenin,[3] ZO-1,[4] and more recently, symplekin.[5]

Symplekin was originally discovered as a component of the tight junction (TJ) cytoskeletal plaques in epithelial and endothelial cells.[6] This protein is also found in the nucleus and, to a lesser degree, in the cytoplasm of cells devoid of TJs.[6] Although no function has been assigned to symplekin at the TJ to date, several studies have shown that it is involved in the nuclear pre-mRNA cleavage and polyadenylation machinery,[7,8] as well as in the polyadenylation of cytoplasmic mRNAs and the maturation of histone mRNAs, which are not polyadenylated.[9–11] Symplekin also interacts directly with transcription factors such as HSF1 and ZONAB/DbpA, thus promoting the transcription of genes involved in the response to stress[12] or in epithelial cell proliferation.[5]

In the present study, we analyzed symplekin expression in human colonic crypts and identified the occurrence of

Abbreviations used in this paper: AML1, acute myeloid leukemia-1; BSA, bovine serum albumin; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HRP, horseradish peroxidase; IgG, immunoglobulin G; KLF4, Krüppel-like factor 4; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; RT-qPCR, reverse transcription–quantitative PCR; shRNA, short hairpin RNA; siRNA, small interfering RNA; TJ, tight junction; YAMC, young adult mouse colon.

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an expression gradient, with high levels of mostly nuclear expression in the proliferative compartment and weaker, mostly membrane-bound levels, in differentiated cells. We then used inducible symplekin down-regulation to determine whether symplekin could behave as a regulator of intestinal differentiation. Our results strongly suggest that symplekin behaves as a negative regulator of differentiation, acting by a repression of the transcription factor Runx1/AML1, which we identify for the first time as a promoter of goblet cell differentiation.

Materials and Methods

Supplemental information (antibody concentrations, sequences of primers used for acute myeloid leukemia-1 [*AML1*] gene recombination and RNA detection) are supplied in Supplementary Table 1.

Cell Culture

Cells were grown as described in Kavanagh et al.[5]

AML-1 Promoter Assay

Cells (6×10^4) per well (96-well plate) were seeded the day before transfection, then transfected in triplicates using 0.1 μL DharmafectDuo (Dharmacon, Lafayette, CO) with 100 nmol/L of control small interfering RNA (siRNA) or ZONAB siRNA together with 98 ng of *AML1* reporter (a generous gift from Dr Motomi Osato, Institute of Molecular and Cell Biology, Proteos, Singapore) and 2 ng of a CMV-Renilla reporter, used as a transfection control. Forty-eight hours after transfection, cells were lysed in 50 μL passive lysis buffer (Promega, Madison, WI), and luciferase activity was measured using the Dual Glow Luciferase Assay System (Promega), according to the manufacturer's instructions.

SiRNA Transfections

HT29-Cl.16E cells were seeded into 6-well plates at 2 × 105 cells/well and transfected the following day with 120 pmol of siRNAs with the use of the EXGEN 500 transfection reagent (Euromedex, Mundolsheim, France). Seventy-two to 96 hours after transfection, cells were harvested, and total RNA was isolated or RIPA protein lysates were prepared, respectively. The SMARTpool siRNA cocktail against ZONAB/DbpA was purchased from Dharmacon (Perbio Science, Brebières, France), and the siRNA cocktail against AML1 was previously described[13] and was from Eurogentec (Angers, France).

Western Blot Analysis

HT29-Cl.16E cells were lysed in 1× RIPA buffer, and Western blot analysis was performed as previously described.[14] Protein concentrations were determined with the use of the DC protein concentration measurement kit (Bio–Rad, Marnes-la-Coquette, France). Total protein lysate (20 μg) was loaded per lane of 7%–10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Proteins were transferred onto polyvinylidene fluoride or nitrocellulose membranes. Detection was performed using the ECL plus system (Amersham, Buckinghamshire, United Kingdom). Optimal exposure times of membranes were used, and protein expression was quantified with the use of the NIH Image 1.62 (National Institutes of Health, Bethesda, MD) and adjusted for background noise and protein loading.

Tissue Samples

Paraffin-embedded sections from macroscopically normal epithelium from 8 patients were obtained from the pathologist after biopsy according to French government regulations and with approval of the ethical committee (Nîmes Hospital).

The intestine of previously described $AML1^{\Delta/\Delta}$ mice[15] was dissected out, rinsed in phosphate-buffered saline (PBS), then separated into 4 segments corresponding to the duodenum, jejunum, ileum, and colon. The lumen was cleaned by gentle PBS flushing, and each segment was rolled and placed into a paraffin-embedding cassette, fixed for 4 hours at room temperature in 4% paraformaldehyde (PFA), rinsed in PBS, and paraffin-embedded with the use of standard procedures.[16] Alternatively, the intestinal epithelium was scraped, and genomic DNA and RNA were prepared with standard procedures. Genomic DNA was analyzed by polymerase chain reaction (PCR) to detect *AML1* recombination, as previously described.[15] To detect expression of the *AML1/Runx1*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Krüppel-like factor 4 (*KLF4*), Muc-2, and *β2-microglobulin* genes, cDNA was prepared from 2.5 μg of RNA with the use of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and all cDNAs were amplified with the use of 29 PCR cycles.

Immunofluorescence

Cells were grown on glass coverslips in 12-well plates until ready for processing, then washed 2 times in PBS, fixed on ice for 30 minutes in 95% ethyl alcohol, and postfixed for 1 minute with acetone. Alternatively, cells were fixed in 3% PFA for 15 minutes at room temperature and permeabilized in PBS/0.2% Tween 20 for 10 minutes. Nonspecific sites were blocked with PBS, 0.5% bovine serum albumin (BSA), 10 mmol/L glycine or PBS, 5% BSA, 0.3% gelatin for 30 minutes. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Secondary antibodies used were goat anti-mouse immunoglobulin G (IgG)–Alexa488 (Molecular Probes, Cergy Pontoise, France) or cyanine 3–coupled goat anti-rabbit IgG (Chemicon, Hampshire, United Kingdom). Samples were mounted in mowiol (Aldrich, Lyon, France). Confocal images were obtained with a Bio-Rad MRC 1024 confocal setup (Optiphot2 microscope, with a Plan Apo 20×/0.75 lens [Nikon, Tokyo, Japan]), and mounted as figures with the use of Photoshop (Adobe Systems, Mountain View, CA).

Muc2 Flow Cytometry

Cells were transfected as described above, and Muc2 detection by flow cytometry was performed as described in Kim et al.[17] Briefly, 72 hours after transfection, cells were resuspended and fixed in 4% PFA for 15 minutes on ice. In the case of cells transfected with AML1–green fluorescent protein (GFP) cDNA, fixation was performed using ice-cold methanol for 10 minutes at −20°C, to minimize GFP fluorescence quenching. Cells were permeabilized with 0.5% Triton X-100, washed, and sequentially incubated with a mouse monoclonal anti-Muc2 antibody (Transduction Laboratories, Lexington, KY) then with a phycoerythrin-coupled donkey anti-mouse IgG antibody. Incubations were performed for 30 minutes at room temperature in the dark and were separated by 3 washes in PBS + 1% BSA. The percentage of phycoerythrin and GFP-positive cells was determined with the use of the BD FACS (fluorescence-activated cell sorting) CantoTM II (Becton Dickinson Biosciences, Franklin Lakes, NJ). A minimum of 10,000 events were analyzed in each sample, and the data were processed with the use of the BD FACS DivaTM software program (BD Biosciences).

Immunohistochemistry

Sections were deparaffinized with standard procedures, stained in a 1% alcian blue solution for 20 minutes, counterstained, and mounted in Entellan (Merck, Darmstadt, Germany). Images were acquired with the use of an Axiophot microscope (Carl Zeiss MicroImaging Inc, Thornwood, NY), 10 × 0.3 Plan Neofluar or 40 × 1.0 Plan Apochromat lenses (Carl Zeiss MicroImaging Inc) and a DXM1200 camera (Nikon). Images were mounted as figures with the use of Photoshop (Adobe Systems).

ChIP Assay

Chromatin immunoprecipitations (ChIP) were essentially done as suggested by the supplier (Upstate, Lake Placid, NY). Briefly, cells were grown to 60% confluence and 1×10^6 cells were cross-linked with 1% formaldehyde. Genomic DNA was sheared, and immunoprecipitations were performed by using 1 μg of anti-AML1 or anti-ZONAB/DbpA antibody at 4°C overnight. Coprecipitated promoter sequences were then analyzed after extraction of the DNA by nested PCR. Primer sequences are supplied in Supplementary Table 1.

Electrophoretic Mobility Shift Assay

Complementary strands of oligonucleotides were annealed and labeled by fill-in reactions with Klenow DNA polymerase and deoxynucleotide triphosphate in the presence of [α-^{32}P]-deoxyadenosine triphosphate. Protein extracts were prepared from cells grown under the conditions indicated in each experiment, as described before.[18] The binding reactions contained, unless indicated otherwise, 8 μg of protein, 1 ng of labeled DNA, and 600 ng of sonicated salmon sperm carrier DNA. In some experiments, an excess of unlabeled ("cold") probe was added to the reactions. The proteins were allowed to bind to the DNA for 15 minutes at room temperature and separated by a nondenaturing 5% polyacrylamide gel for approximately 3 hours at 110 V. The gel was then dried and exposed onto a PhosphorImager screen (GE Healthcare, Rydalmere NSW, Australia), scanned, and analyzed with the use of the ImageQuant software package (GE Healthcare). The positive control for binding to AML-1/RunX1 was described before.[18] Oligonucleotide probe sequences are provided in Supplemental Table 1.

Results

Symplekin Expression Pattern in the Human Colon

We first analyzed the expression pattern of symplekin in the human colonic epithelium. With the use of 2 different symplekin antibodies recognizing, respectively, the nuclear and membrane-associated forms of symplekin,[6,7] we found that nuclear expression of symplekin was not homogenous along the crypt-villus axis, with the highest quantities expressed in cells located in the proliferating area, at the bottom of Lieberkühn crypts. Membrane symplekin was mostly concentrated in the TJ area, with weak staining also detectable along the lateral membrane. Similar staining was found in the colon epithelium of all 8 samples analyzed (Figure 1*A*; Supplemental Figure 1).

Symplekin Depletion Facilitates Cell Differentiation Along the Goblet Cell Lineage

To determine whether symplekin was capable of modulating the differentiation of intestinal cells, we used the 16E subclone of HT29 colorectal cells, which is committed toward goblet cell differentiation.[19] As shown in Figure 1*B*, tetracycline-inducible, short hairpin RNA (shRNA)–mediated symplekin down-regulation, previously described in this model,[5] strongly increased the expression of mRNA encoding the intestinal goblet cell marker Muc2 in HT29-Cl.16E cells. Accordingly, the intensity and number of mucin-producing, alcian blue–positive cells (Figure 1*C*), and of cells positively stained with a selective antibody against Muc2 (Figure 1*D*), were increased in clones in which symplekin expression was down-regulated for 5 days (Figure 1*D*). Electron microscopic observation of cells labeled with horseradish peroxidase (HRP)–coupled Muc2 antibodies showed that this effect indeed corresponded to an increased presence of fully differentiated, mucus-secreting goblet cells (Figure 1*E*). In contrast, mRNAs for the enterocyte-specific marker alkaline phosphatase were barely detectable by reverse transcription–quantitative PCR (RT-qPCR) both in HT29Δsym cells and in controls, confirming that differentiation toward the absorptive cell lineage is not promoted in these cells (not shown).

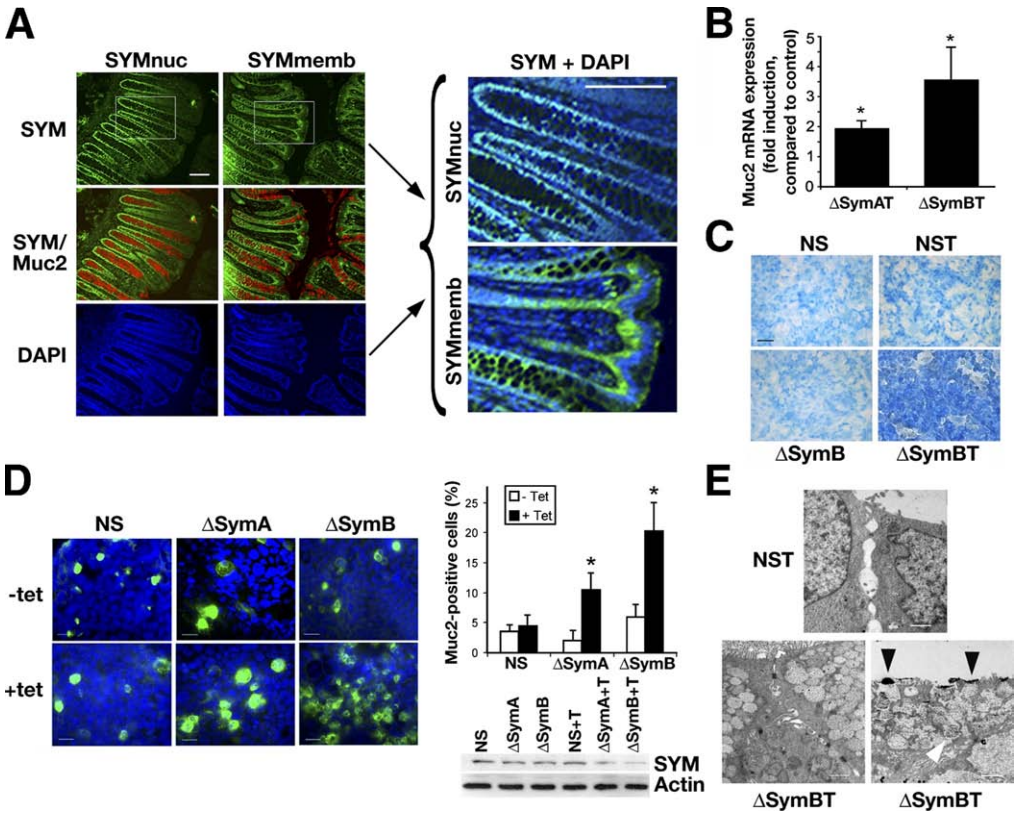

Figure 1. Symplekin depletion promotes goblet cell differentiation. (*A*) Representative micrograph showing expression of nuclear (SYMnuc) and membrane-associated (SYMmemb) symplekin in sections of macroscopically healthy human colonic mucosa (in *green*). Goblet cells were detected with Muc-2 staining (in *red*), and nuclei were stained with DAPI (in *blue*). Similar staining was obtained in colon epithelium samples obtained from 8 patients. Another representative example from a different patient is shown in Supplementary Figure 1. (*B*) RT-qPCR quantification of mRNAs prepared from HT-29Cl.16E cells expressing independent shRNAs directed against different regions of the symplekin mRNA (ΔSymA and ΔSymB) and grown in the presence of tetracycline (tet) for 14 days after confluence, using Muc2-specific primers. Values were corrected for total RNA expression using GAPDH mRNA and are expressed as fold-induction of Muc2 mRNA in ΔSymA and ΔSymB compared with NS control cells after tet treatment. (*C*) Alcian blue staining of ΔSymB cells grown in the presence or absence of tetracycline for 5 days (T). *Scale bar*, 20 μm. (*D left*) Immunofluorescence staining of Muc2 in NS, ΔSymA, and ΔSymB cells, grown in the presence or absence of tetracycline for 5 days. *Scale bar*, 20 μm. (*Right top*) *Histogram* summarizing the percentage of Muc2-positive cells in NS, ΔSymA, and ΔSymB populations. (*Right bottom*) Protein lysates from control, ΔSymA, and ΔSymB cells were analyzed for symplekin expression with Western immunoblotting. Actin was used as a loading control. (*B* and *D*) Results are expressed as mean ± SEM from 3 independent experiments ($P < .05$ compared with control cells; *Student's *t* test). (*E*) Representative micrographs of transmission electron microscopy analysis of control (NS) and ΔSym cells grown for 14 days after reaching confluence in the presence of tetracycline (T). (*Right*) Black precipitates after immuno-HRP staining of Muc2, reflecting mucin secretion (*black arrowheads*) as well as presence of mucin within secreting vesicles (*white arrowhead*). *Scale bar*, 1 μm.

These results show that the down-regulation of symplekin significantly increases the number of Muc-2–positive goblet cells and the production of mucins by individual cells.

AML1/Runx1-Mediated Up-Regulation of KLF4 Leads to Muc2 Increase After Symplekin Down-Regulation

To understand the promoting effect of symplekin down-regulation on goblet cell differentiation, we quantified the expression of several transcription factors previously shown to be involved in intestinal differentiation. We found that HT29Δsym treated with tetracycline for 5 days expressed very high levels of KLF4 (Figure 2*A*), a transcription factor shown to be required for terminal differentiation of goblet cells in the colon.[20] In contrast, expression levels of *HES-1* and *HATH-1* were not significantly modified in these cells (data not shown), suggesting that increased KLF4 expression was not due to a modulation of the Notch pathway downstream of symplekin. In silico analysis of the KLF4 promoter showed the presence of 2 consensus sites for the transcription factor AML1/Runx1, which we also found to be up-regulated in a gene expression array performed after symplekin down-regulation in HT29 cells (not shown). We then confirmed by RT-qPCR and Western blotting that AML1 was expressed by HT-29-Cl.16E cells and that it was up-regulated after symplekin down-regulation (Figure 2*B*). To determine whether KLF4 overexpression depended on increased AML1 levels, a human AML1/Runx1 construct was transiently transfected into HT29-Cl.16E cells. Within the next 72 hours, exogenous AML1 expression was indeed sufficient to increase significantly the expression of KLF4 and, to a lesser degree, Muc2 mRNA (Figure 2*C*). Similar results were found at the protein level

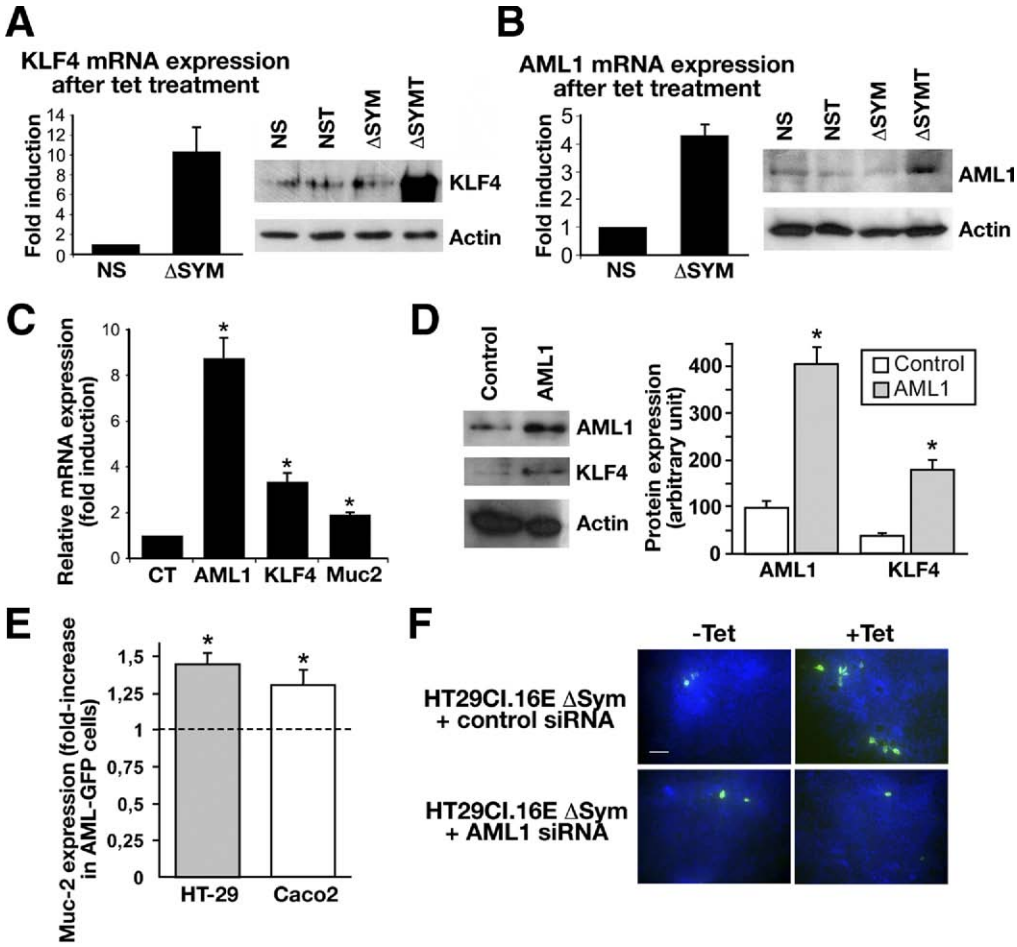

Figure 2. AML1/Runx1 up-regulation is responsible for increased KLF4 expression in HT29ΔSym cells. (*A, left*) Relative expression of KLF4 mRNA in tetracycline-induced NS and ΔSYM clones, quantified with RT-qPCR; (*A, right*) representative Western immunoblotting showing an increase in KLF4 protein expression 5 days after symplekin down-regulation (ΔSYMT). (*B, left*) Relative expression of AML1/Runx1 mRNA in tetracycline-induced NS and ΔSYM clones, quantified with RT-qPCR; (*B, right*) detection of AML1 protein expression by immunoblotting 5 days after symplekin down-regulation (ΔSYMT). (*C*) AML1/Runx1, KLF4, and Muc2 mRNA levels were quantified in HT29-Cl.16E cells overexpressing a human AML1 construct. Results are expressed as fold-induction compared with the respective expression level of each of these genes in control HT29-Cl.16E cells (CT). Values are mean ± SEM from 3 independent experiments (*$P < .05$ compared with controls, Student's *t* test). (*D, left*) AML1 and KLF4 expression in HT29-Cl.16E cells transfected with control or AML1 constructs was measured with immunoblotting. (*D, right*) Quantification of AML1 and KLF4 protein levels from 3 independent immunoblotting experiments, using actin as a loading control. (*$P < .05$ compared with control cells, Student's *t* test). (*E*) Quantification of Muc2 expression with flow cytometry in HT29-Cl.16E and Caco2 cells transiently transfected with pEGFP or AML1-GFP constructs. The proportion of GFP-positive cells that were staining for Muc2 was quantified 72 hours after transfection in each population, and results represent the fold-increase of Muc2 staining in AML-1–GFP cells compared with pEGFP cells (*$P < .05$, Student's *t* test; n = 3). Expression level in pEGFP is represented by the *dashed line*. (*F*) Muc2 staining of HT29-Cl.16E ΔSym cells, treated (+Tet) or not (−Tet) with tetracycline for 5 days, after transfection with β-galactosidase (control) or AML1-specific siRNA. *Scale bar*, 50 μm.

for KLF4 (Figure 2*D*), and Muc2 in HT29-Cl.16E and in Caco2 colorectal cancer (CRC) cells (Figure 2*E*). In addition, siRNA-mediated AML1 down-regulation prevented the strong increase in goblet cell differentiation normally seen after symplekin down-regulation (Figure 2*F*). These results indicate that AML1 is an intermediate for the regulation of intestinal goblet cell differentiation by symplekin.

With the use of ChIP assay, we then showed that the *KFL4* promoter was detected in AML1 immunoprecipitates after tetracycline-induced symplekin down-regulation in HT29-Cl.16E cells (Figure 3*A*), and electrophoretic mobility shift assays (EMSAs) showed that AML1 was binding to only 1 of the 2 putative sites detected within the *KLF4* promoter (Figure 3*B–D*). These results strongly suggest that *KLF4* is a direct transcriptional target of AML1/Runx1 in intestinal cells.

AML1/Runx1 Promotes Intestinal Goblet Cell Differentiation In Vivo

To confirm the physiologic relevance of the AML1 role in intestinal goblet cell differentiation, we quantified the number of goblet cells in the intestinal epithelium of mice carrying a loxP-flanked exon 5 of the *AML1* gene (*AML1$^{fl/fl}$*), allowing recombination under the control of an interferon-responsive Mx-promoter, after PolyIPolyC treatment.[15] Because several AML1 antibodies proved in-

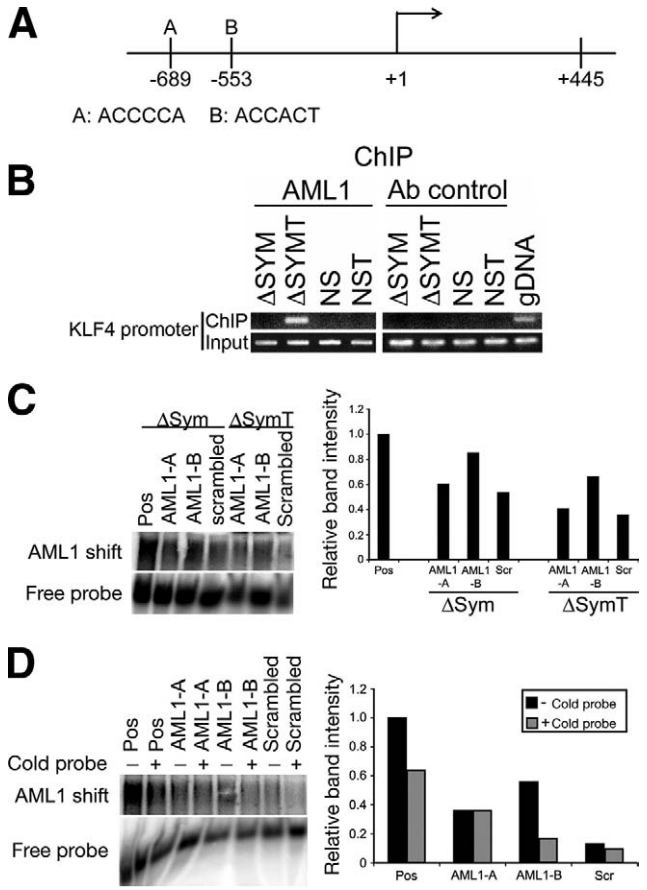

Figure 3. AML1/Runx1 preferentially binds to 1 of its 2 putative binding sites on the human *KLF4* promoter. (*A*) Representation of the human *KLF4* promoter, depicting the transcription and translation initiation sites and the position and sequences of the 2 putative AML1/Runx1 binding sites. (*B*) Detection of the *KLF4* promoter region containing the putative AML1 binding site after AML1 immunoprecipitation. The KLF4 promoter sequence present in immunoprecipitation samples and input controls was amplified by nested PCR and analyzed by agarose gel electrophoresis after ChIP with AML1 antibody or control antibody, on cross-linked lysates of ΔSYM and control clone (NS). gDNA indicates genomic DNA (positive control for nested PCR). (*C*) The capacity of AML1/Runx1 to bind to segments A or B of the *KLF4* promoter was assessed by an EMSA, using the human Ada gene[19] and a scrambled *KLF4* promoter sequence as positive and negative controls, respectively. (*D*) The specificity of AML1/Runx1 binding to either of its 2 sites on the *KLF4* promoter was controlled, by using the respective unlabeled probes as competitors in the EMSA. Binding of AML1 to site A is not shifted by the unlabeled probe, indicating that it is most likely nonspecific.

effective in our hands to detect this protein with immunohistochemistry in mouse tissues, we used RT-qPCR analysis of epithelial samples from the duodenum, jejunum, ileum, and colon of 1-year-old mice to show that AML1/Runx1 transcripts are expressed throughout the murine intestinal epithelium and that their expression is markedly reduced in the intestine of AML1$^{\Delta/\Delta}$ mice (Figure 4*A*). PCR of intestinal genomic DNA[15] showed that recombination of the *AML1/Runx1* gene was detected in all 4 segments of the intestine in AML1$^{\Delta/\Delta}$ mice only, albeit at a lower efficiency than in bone marrow samples (Figure 4*A*). The intestine of these mice appeared morphologically normal and did not display any signs of colitis (data not shown), but alcian blue staining showed that goblet cell numbers were reduced by 30%–40% throughout the intestine of AML1$^{\Delta/\Delta}$ mice (Figure 4*B*). In

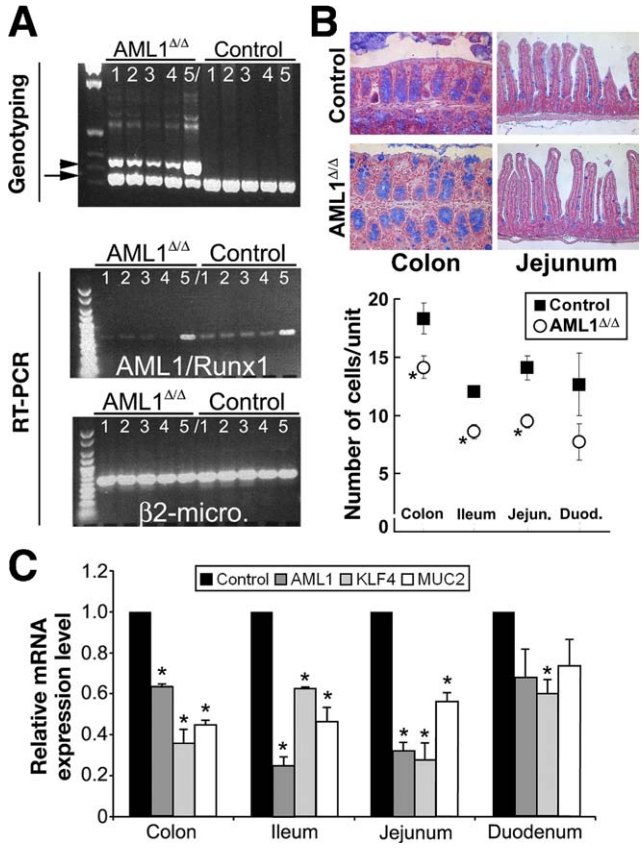

Figure 4. AML1 is a positive regulator of goblet cell terminal differentiation in vivo. (*A*) Recombination and down-regulation of AML1/Runx1 expression in the intestine of AML$^{\Delta/\Delta}$ mice. (*Top*) Partial recombination of the Runx1 gene was detected by PCR in the intestinal epithelium (*lanes 1–4*) and the bone marrow (*lane 5*) of AML$^{\Delta/\Delta}$ mice (*black arrowhead*), whereas control animals only displayed the wild-type allele (*black arrow*). Representative gels from one animal of each group are shown (4 AML$^{\Delta/\Delta}$ and 4 control mice were analyzed in total). (*Bottom*) Expression of AML1/Runx1 (*top*) and β2-microglobulin (*bottom*) transcripts in the intestine (*lanes 1–4*) and bone marrow (*lane 5*) of AML1$^{\Delta/\Delta}$ and control mice. *Lane 1* is *duodenum*; *lane 2*, *jejunum*; *lane 3*, *ileum*; and *lane 4*, *colon*. (*B top*) Representative microphotographs of colon and jejunum epithelial sections from control and AML1$^{\Delta/\Delta}$ mice, after alcian blue staining of goblet cells and nuclear fast red counterstaining. (*Bottom*) Alcian blue staining was performed on paraffin-embedded section of epithelium from the duodenum, jejunum, ileum, and colon of control (*black squares*) and AML1$^{\Delta/\Delta}$ mice (*white circles*), and goblet cell numbers were counted in multiple fields representing 200–250 crypt/villus units per animal. *$P < .05$ compared with goblet cell numbers in the corresponding tissue in control animals, Student's *t* test (n = 4 mice/group). (*C*) RNA lysates were prepared from the colon, ileum, jejunum, and duodenum of control and AML1$^{\Delta/\Delta}$ mice and were analyzed for the expression of AML1/Runx1, KLF4, and Muc2 mRNA, using RT-qPCR. For each gene, the expression level in AML1$^{\Delta/\Delta}$ mice is pictured relative to the level in control animals, which was arbitrarily set to 1 (*black bars*). Results are mean ± SEM from 2 separate experiments on 4 mice per group. (*$P < .05$ compared with control cells, *t* test).

addition, mRNA quantification confirmed that AML1, KLF4, and Muc2 levels were down-regulated in the intestinal epithelium of AML1$^{\Delta/\Delta}$ mice (Figure 4*C*). The strongest decrease in goblet cell numbers and AML1, KLF4, and Muc2 mRNA levels was found in the jejunum, ileum, and colon (Figure 4*B* and *C*). Symplekin levels were not affected in the intestine of AML1$^{\Delta/\Delta}$ mice compared with controls (data not shown), indicating that the effect of AML1 deletion was not due to a feedback regulation on symplekin expression.

These results show that AML1 is a positive regulator of KLF4 expression and of goblet cell terminal differentiation in the murine intestine in vivo.

AML1/Runx1 Expression Is Repressed by the Symplekin/ZONAB/DbpA Complex in Intestinal Cells

Finally, we sought to determine how symplekin was able to regulate negatively the expression of AML-1 in intestinal cells. Recent results from Kavanagh et al[5] and us showed that symplekin interacts with the Y-box transcription factor ZONAB/DbpA to regulate gene expression in intestinal cells. Here, we identified a consensus site for ZONAB/DbpA in the *AML1* promoter, and ChIP experiments showed that the *AML1* promoter could be coimmunoprecipitated with anti-ZONAB/DbpA antibodies from HT29-Cl.16E cell lysates and that the amount of ZONAB/DbpA bound to the *AML1* promoter was decreased in HT29ΔSym clones (Figure 5*A*). This result may be due to the reduced nuclear localization of ZONAB after symplekin down-regulation (Kavanagh et al[5]; Supplemental Figure 2). siRNA-mediated down-regulation of ZONAB/DbpA also induced a strong increase in the expression of AML1 and KLF4 mRNA (Figure 5*B*) and AML1 protein (Figure 5*C*) in HT29-Cl.16E cells, resulting in a significant increase in the number of alcian blue– and Muc-2–positive cells (Figure 5*D*), similar to what was found in HT29ΔSym clones (Figure 1*A*). Down-regulation of ZONAB/DbpA also increased the expression of Muc2 in Caco2 CRC cells, as well as in the nontumoral mouse intestinal cell lines young adult mouse colon (YAMC)[21] and Apc$^{+/+}$[22] (Supplemental Figure 3).

Finally, the activity of a minimal AML1/Runx1 promoter was quantified with a luciferase reporter assay in HT29-Cl.16E/ΔSym cells transfected with a ZONAB-selective siRNA and treated or not with tetracycline (Figure 5*E*). The activity of this promoter was significantly increased on symplekin down-regulation (+tet) in cells expressing the control siRNA, as well as on treatment of noninduced cells (−tet) with ZONAB siRNA. In contrast, siRNA-mediated ZONAB down-regulation did not further increase AML1/Runx1 reporter activity in induced HT29-Cl.16E/ΔSym cells, strongly suggesting that ZONAB binding to the *AML1* promoter is essential for the effect of symplekin on AML1 expression (Figure 5*E*).

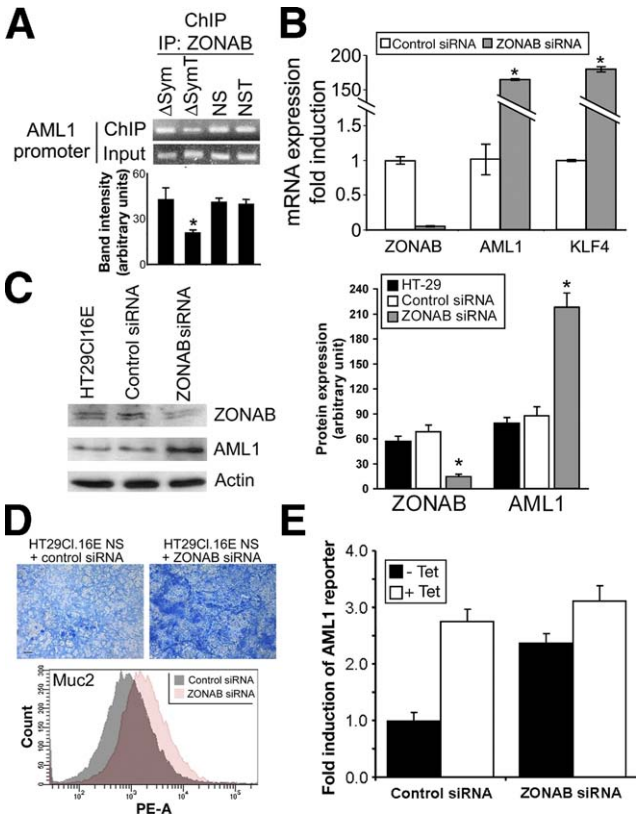

Figure 5. ZONAB/Dpba is a negative regulator of AML1/Runx1. (*A, top*) ChIP of ZONAB/Dpba binding site in the AML1/Runx1 promoter from cross-linked lysates of ΔSym and control clone (NS), treated or not with tetracycline for 5 days, using an anti-ZONAB/Dpba antibody. The AML1/Runx1 promoter sequence present in the immunoprecipitation samples and input controls was amplified by nested PCR and analyzed by agarose gel electrophoresis. (*A, bottom*) *Histogram* showing relative band intensities of immunoprecipitation samples, after correction for input band intensity. Values are mean ± SEM from 3 independent experiments. Note that the amount of AML1 promoter coimmunoprecipitating with ZONAB/Dpba is significantly reduced in cells with down-regulated symplekin. *$P < .05$ compared with untreated ΔSym cells and to control cells. (*B*) Real-time quantitative PCR analysis of ZONAB/Dpba, AML1/Runx1, and KLF4 mRNA expression levels in HT29-Cl.16E cells transfected with β-galactosidase–specific (control, *white bars*) or ZONAB/Dpba-specific (*gray bars*) siRNA for 72 hours. (*C, left*) Western immunoblotting detection of ZONAB/Dpba, AML1, and actin (loading control) protein expression levels, 72 hours after transfection of HT29-Cl.16E cells with ZONAB/Dpba or control siRNA. (*C, right*) Quantification of ZONAB and AML1 protein levels from 3 independent Western immunoblotting experiments, using actin as a loading control. (*$P < .05$ compared with control cells, Student's *t* test). (*D*) Alcian blue staining (*top*) and Muc-2 flow cytometry (*bottom*) on HT29-Cl.16E control cells (NS) after transfection with β-galactosidase (control) or ZONAB/Dpba-specific siRNA. *Scale bar*, 20 μm. (*E*) HT29-Cl.16E/ΔSym cells were cotransfected with the AML1 gene promoter fused to Luciferase and with the control or ZONAB-selective siRNA, then treated or not with tetracycline after 48 hours, and promoter activity was quantified as described in "Materials and Methods." Results are mean ± SEM from 3 independent experiments (*$P < .05$ compared with untreated cells with control siRNA, Student's *t* test).

Taken together, these results indicate that nuclear symplekin and ZONAB/DbpA cooperate to maintain low levels of AML1 expression in intestinal cells, thereby negatively modulating their differentiation along the goblet cell lineage.

Discussion

In the present work, we detected a strong nuclear expression of symplekin in the proliferative compartment of colonic crypts within the healthy epithelium, whereas its expression becomes weaker and predominantly membrane associated in terminally differentiated cells. We also report for the first time that symplekin acts as a negative modulator of intestinal goblet cell differentiation. In addition, we identify AML1/Runx1 as a key intermediate of this effect and show that AML1 is a promoter of intestinal goblet cell differentiation in vitro and in vivo, acting by the transcriptional activation of KLF4. In addition, our results indicate that AML1 expression is down-regulated by the symplekin/ZONAB complex in intestinal cells.

The symplekin expression pattern detected in the first part of this work, characterized by a strong nuclear expression of symplekin in the proliferative compartment of colonic crypts, is in agreement with previous results, suggesting that symplekin behaves as a transcriptional modulator, cooperating with the transcription factor ZONAB/DbpA in the nucleus to control the expression of genes involved in proliferation.[5] Here, we also show that down-regulating symplekin expression in HT29 cells promotes their differentiation toward the goblet cell phenotype, suggesting that symplekin also acts as a repressor of differentiation in these cells. We found that symplekin was able to repress the expression of *AML1/Runx1*, originally identified as a gene located at the breakpoint of the t(8;21) translocation, and which encodes a transcription factor behaving as an essential regulator of hematopoiesis.[23] Although Runx family members have been implicated in the transition between proliferation and differentiation in other organs,[24] the present work provides the first description of a role for AML1/Runx1 in the differentiation of Muc-2–producing goblet cells in the murine intestine in vivo. This result provides a possible explanation to results obtained by Nam et al,[25] who found that *run*, the nematode ortholog of Runx factors, was involved in the development of a functional gut in C. *elegans*, even though the lack of major morphologic alterations in the intestinal mucosa of $AML1^{\Delta/\Delta}$ mice indicates that intestinal architecture is preserved in the absence of AML1. In addition, symplekin down-regulation promoted goblet cell differentiation by an AML1-dependent increase of KLF4 expression in HT29 cells. In agreement with our data, KLF4 was shown to be essential for the differentiation of goblet cells in the mouse intestine.[20] The fact that the reduction of goblet cell numbers in $AML1^{\Delta/\Delta}$ mice is less drastic than that observed in animals in which the *KLF4* gene is invalidated is likely to reflect differences in tissue-specific Cre-recombinase activity. However, AML1 could also be involved in one of several complementing pathways converging to control KLF4 expression and terminal maturation of goblet cells.

Although a large amount of work has been devoted to studying the AML1-ETO fusion protein in acute myeloid leukemia and the biological role of AML1 in the proliferation or differentiation of various cell types,[24–26] surprisingly little is known about its transcriptional regulation. AML1 can promote its own expression,[26] and the level of AML1 in hematopoietic stem cells is increased by Bmp4 during mouse embryonic development, by the binding of its transducer Smad1 to the AML1 promoter.[27] In the present work, we identify for the first time that the transcriptional regulator ZONAB/DbpA binds to the *AML1* promoter and that symplekin and ZONAB/DbpA negatively regulate AML1 expression. Accordingly, our results strongly suggest that ZONAB/DbpA cooperates with nuclear symplekin to repress differentiation, similar to what was recently described for its role in the promotion of epithelial cell proliferation.[5] Indeed, siRNA-mediated down-regulation of the Y-box transcription factor ZONAB/DbpA in several intestinal cell lines triggered a similar differentiation process to that found in cells in which symplekin expression was down-regulated. Although Caco-2 cells have previously been shown to express the goblet cell marker Muc-2,[28,29] Caco-2, YAMC, and $APC^{+/+}$ cells are not "primed" for goblet cell lineage differentiation as HT-29 cells are, suggesting that the regulatory mechanism involving symplekin and ZONAB acts upstream of terminal goblet cell differentiation.

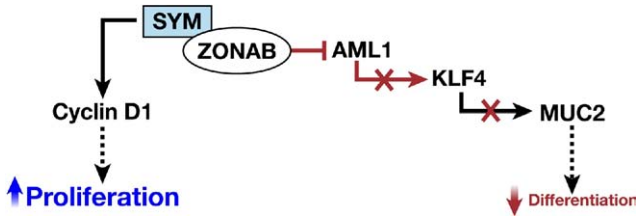

Figure 6. Nuclear symplekin acts as an activator of proliferation and as a negative regulator of intestinal cell differentiation. Symplekin cooperates with ZONAB/DbpA to increase the transcription of cyclin D1[5,31] and to down-regulate the expression of AML1, KLF4, and Muc2, resulting in increased proliferation and reduced goblet cell differentiation. *Red arrows* and *crosses* represent results obtained in the present study.

In conclusion, we propose a model (Figure 6) whereby, along with its previously shown role as an activator of proliferation, the symplekin/ZONAB complex acts as a negative regulator of intestinal cell differentiation, acting by the repression of AML1/Runx1 levels in undifferentiated cells. In cells with lower levels of symplekin, AML1/Runx1 upregulation in turn promotes differentiation along the secretory lineage by directly controlling the expression of KLF4, a factor well known for its role in goblet cell differentiation. Along with a recent report showing that biallelic inactivation of Runx3 induces colon adenomas in the absence of nuclear β-catenin accumulation,[30] the present data highlight the emerging importance of this gene family in the control of intestinal homeostasis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of

Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.03.037.

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Conflicts of interest

The authors disclose no conflicts.

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Supplemental Material and Methods

Antibody Concentrations for Western Blotting

Antibody concentrations were: anti-symplekin (Becton Dickinson, 1/5000), anti-Actin (Clone AC-40, Sigma, 1/4000), anti-ZONAB/DbpA (Zymed, 1/2000), anti-AML1 (Sigma, 1/1000), and anti-KLF4 (Santa Cruz Biotechnology, 1/3000). HRP-coupled secondary antibodies were from Chemicon (Hampshire, UK).

Antibodies

Antibodies against ZONAB/DbpA were purchased from Zymed (InVitrogen, Cergy Pontoise, France). Anti-Symplekin antibodies were from Becton Dickinson (Le Pont-de-Claix, France). Anti-Muc 2 was from Neomarkers, Lab Vision (UK) Ltd, (Newmarket, UK). Antibodies against actin and AML1/Runx1 were from Sigma (St. Quentin Fallavier, France). Anti-KLF4 was from Santa Cruz Europe (Heidelberg, Germany). Antibodies against the transcription factors, AML1 and KLF4, were found to detect their respective protein targets only after mild detergent treatment of proteins (WB, ChIP), but did not provide any positive staining when used for immunohistochemistry on mouse or human tissues, even after heat-induced epitope unmasking.

A) Primer Sequences for Quantitative RT-PCR on HT29Cl.16E Cells

Gene Name
Forward Primer
Reverse Primer

GAPDH
GAGAAGGCTGGGGCTCAT
TGCTGATGATCTTGAGGCTG

SYMPK
TGAGTTCCTGCAGCCTCTG
CTCCACGGGGGTGTAGATG

MUC2
CATCTGTTCCATTACGACACG
GGTGGTGGTGGTAGTGGTG

KLF4
CCCATCTCAAGGCACACC
GCATTTTTGGCACTGGAAC

RUNX1 (AML1)
TCCCTGAACCACTCCACTG
GATGGTTGGATCTGCCTTGT

ZONAB/DbpA
GCTGGGGAGGAGGAGGA
CTGTTGGGATGGGGTAAGAC

B) Primer Sequences for ChIP Nested PCR

Promoter/Tx factor binding site
Forward Primer
Reverse Primer

RUNX1/ZONAB
Outside pair
CCCCCTCTTGCAAAGTCTAC
TGACCACTATGCTGGGTTCA
Inside pair
CGGCTATTTTCTTGCACAGC
GAAGTTTTCACACAACCCAAA

KLF4/RUNX1
Outside pair
ATAATCGCGCTCTTCTCCAG
TCGTTGCTATGGCAGCTAAA
Inside pair
CGGCAAGCGCGTATGCTA
GCGGGGGAGGGGAAGGAG

C) Primer Sequences for AML1 Genomic DNA Recombination

Primer 5 (TIMER-FLP2): TAGGGAGTGCTGCTTGCTCT
Primer 6 (TIMER-FLP1): GCCGGGTGCAATATTAAGTC
Primer 7 (AML-CRE1): CTCTGGGAAACCAGGGAGTG

D) Primer Sequences for AML1/Runx1 cDNA Amplification

Runx1 exon 4/5-fw: AGCGACACCCATTTCACC
Runx1 exon 4/5-rev: GGAGATGGACGGCAGAGTAG

mouse B2m-fw: GAGCCCAAGACCGTCTACTG
mouse B2m-rev: GCTATTTCTTTCTGCGTGCAT

E) Primer Sequences for Quantitative RT-PCR on AML1−/− Mouse Tissue

Gene Name
Forward Primer
Reverse Primer

GAPDH
TGG CAA AGT GGA GAT TGT TGC C
AAGATGGTGATGGGCTTCCCG

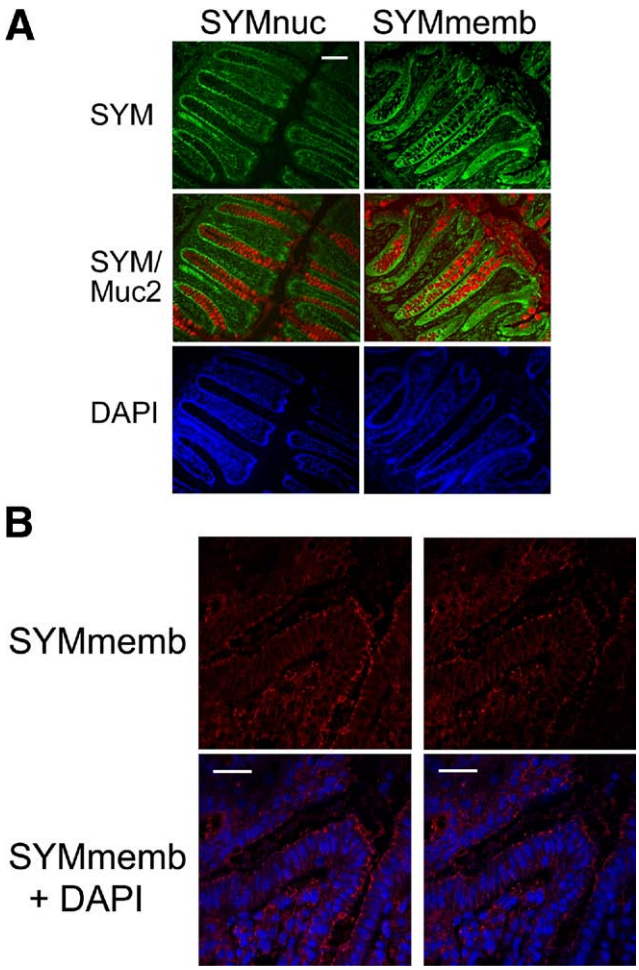
KLF4
CGGGAAGGGAGAAGACACT
GAGTTCCTCACGCCAACG

RUNX1 (AML1)
CTCCGTGCTACCCACTCACT
ATGACGGTGACCAGAGTGC

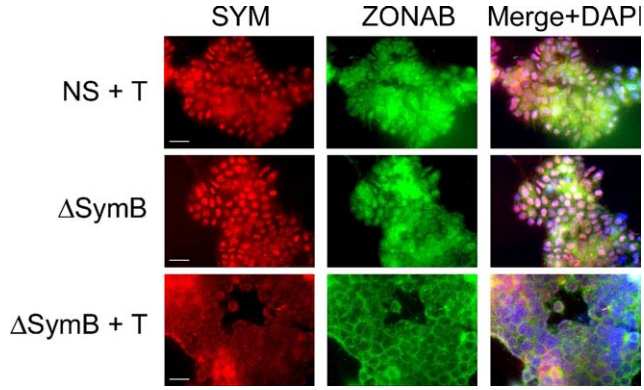
MUC2
CTTCTGTGCCACCCTCGT
TTCGGGATCTGGCTTCTTT

F) Oligonucleotides Used for Electrophoretic Mobility Shift Assay

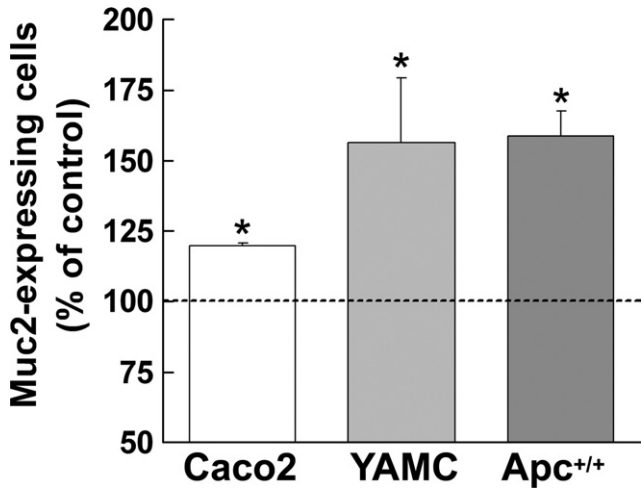
Probe Name	Sense Oligo	Antisense Oligo
AML1 POS	CAGTGGCTCATCGATTGTGGTTCTTGTGC	CAGTGCACAAGAACCACAATCGATGAGCC
AML1-A	CAGTCGCCCGCTGACCCCACCAGTCTTCG	CAGTCGAAGACTGGTGGGGTCAGCGGGCG
AML1-B	CAGTCGCCGCCGACACCACTGCCGCCGGC	CAGTGCCGGCGGCAGTGGTGTCGGCGGCG
AML1 SCR	CAGTGGAAGATAAACTTAATGATCTTTG	CAGTCAAAGATCATTAAGTTTATCTTCC



Supplementary Figure 1. Symplekin expression in the human colonic epithelium. (*A*) Representative micrograph showing expression of nuclear (SYMnuc) and membrane-associated (SYMmemb) symplekin in sections of macroscopically healthy human colonic mucosa (in *green*). Areas of differentiated cells were marked with Muc-2 staining (in *red*), and nuclear DAPI staining is shown in *blue*. (*B*) High magnification confocal sections showing membrane-associated symplekin expression in the upper section of human colonic crypts (in *red*). Nuclei are visualized with DAPI staining. Images were obtained with a Leica Sp2 confocal microscope, using a ×40 oil immersion lens. Two 1-μm sections are shown. *Bars* = 40 μm.



Supplementary Figure 2. Nuclear ZONAB expression is reduced after symplekin down-regulation in HT29-Cl.16E ΔSym cells. The localization of symplekin (SYM, *red*) and ZONAB (*green*) was examined with immunofluorescence staining in control (NS) and ΔSymB cells without or with (+T) tetracycline treatment. Nuclei were visualized in the merged images with DAPI staining. *Bars* = 20 μm. These results are similar to those described in Kavanagh et al.[4]



Supplementary Figure 3. The number of Muc2-expressing cells was quantified with flow cytometry in Caco2, YAMC, and Apc$^{+/+}$ cells transfected with siRNA directed against ZONAB/DBpA or β-galactosidase (Control) as described in "Materials and Methods." Results are expressed as a percentage of the expression detected in cells transfected with control siRNA (the level in control cells is materialized by the *horizontal hashed line*). Values are mean ± SEM from 3 independent experiments (*$P < .05$ compared to control cells, Student's *t* test).