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-CI.16E cells that expressed inducible symplekin short hairpin RNA or were transfected with ZONAB small interfering RNAs. Intestines of AML1H9004 mice were stained with alcian blue and analyzed for expression of AML1/Runx1, GAPDH, KLF-4, and Muc-2. 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-CI.16E colorectal carcinoma cells into goblet cells. Down-regulation of symplekin or 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-CI.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 AML1H9004 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. 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) or as downstream effectors of specific signaling pathways (eg, ephrins/Eph Receptors, E-cadherin/β-catenin). 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, p120 catenin, ZO-1, and more recently, symplekin.

Symplekin was originally discovered as a component of the tight junction (TJ) cytoskeletal plaques in epithelial and endothelial cells. This protein is also found in the nucleus and, to a lesser degree, in the cytoplasm of cells devoid of TJs. 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, as well as in the polyadenylation of cytoplasmic mRNAs and the maturation of histone mRNAs, which are not polyadenylated. 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 or in epithelial cell proliferation.

In the present study, we analyzed symplekin expression in human colonic crypts and identified the occurrence of...
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 × 10^5 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 AML1 was previously described13 and was from Eurogentec (Angers, France). The siRNA cocktail against ZONAB/DbpA was purchased from 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.

**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/−/− mice15 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 postfixied 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 were used 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. 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 FACSM (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 FACSDivaTM software program (BD Biosciences).

**Immunobistochemistry**

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 Axioskop 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 × 10⁶ 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-³²P]-deoxyadenosine triphosphate. Protein extracts were prepared from cells grown under the conditions indicated in each experiment, as described before. 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. 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, 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 1A; 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. As shown in Figure 1B, tetracycline-inducible, short hairpin RNA (shRNA)–mediated symplekin down-regulation, previously described in this model, 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 1C), and of cells positively stained with a selective antibody against Muc2 (Figure 1D), were increased in clones in which symplekin expression was down-regulated for 5 days (Figure 1D). 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 1E). 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).
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 2A), 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 2B). 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 2C). Similar results were found at the protein level.

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. Results are expressed as mean ± SEM from 3 independent experiments (*Student’s t test).
for KLF4 (Figure 2D), and Muc2 in HT29-Cl.16E and in Caco2 colorectal cancer (CRC) cells (Figure 2E). In addition, siRNA-mediated AML1 down-regulation prevented the strong increase in goblet cell differentiation normally seen after symplekin down-regulation (Figure 2F). 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 KLF4 promoter was detected in AML1 immuno-precipitates after tetracycline-induced symplekin down-regulation in HT29-Cl.16E cells (Figure 3A), 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 3B–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<sup>fl/fl</sup>), allowing recombination under the control of an interferon-responsive Mx-promoter, after PolyI PolyC treatment. Because several AML1 antibodies proved in-
Assessed by an EMSA, using the human Ada gene and a scrambled A. PCR of intestinal genomic DNA showed that recombination of the AML1/Runx1 to bind to segments A or B of the KLF4 genomic DNA (positive control for nested PCR). (C) Cross-linked lysates of /H9004 electrophoresis after ChIP with AML1 antibody or control antibody, on promoter sequence present in immunoprecipitation samples and input (SYM) 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 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.

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 /H9004 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 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−/− mice (Figure 4A). PCR of intestinal genomic DNA showed that recombination of the AML1/Runx1 gene was detected in all 4 segments of the intestine in AML1−/− mice only, albeit at a lower efficiency than in bone marrow samples (Figure 4A). 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−/− mice (Figure 4B). In
addition, mRNA quantification confirmed that AML1, KLF4, and Muc2 levels were down-regulated in the intestinal epithelium of AML1ΔΔ mice (Figure 4C). The strongest decrease in goblet cell numbers and AML1, KLF4, and Muc2 mRNA levels was found in the jejunum, ileum, and colon (Figure 4B and C). Symplekin levels were not affected in the intestine of AML1ΔΔ 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 AML1 in intestinal cells. Recent results from Kavanagh et al. 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 5A). This result may be due to the reduced nuclear localization of ZONAB after symplekin down-regulation (Kavanagh et al.; Supplemental Figure 2). siRNA-mediated down-regulation of ZONAB/DbpA also induced a strong increase in the expression of AML1 and KLF4 mRNA (Figure 5B) and AML1 protein (Figure 5C) in HT29-Cl.16E cells, resulting in a significant increase in the number of alcaline blue- and Muc-2-positive cells (Figure 5D), similar to what was found in HT29ΔSym clones (Figure 1A). 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) and ApcΔ/Δ (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 5E). The activity of this promoter was significantly increased on symplekin down-regulation (+tetr) in cells expressing the control siRNA, as well as on treatment of noninduced cells (−tetr) 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 5E).

**Figure 5.** ZONAB/DbpA is a negative regulator of AML1/Runx1. (A, top) ChIP of ZONAB/DbpA 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/DbpA 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/DbpA 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/DbpA, AML1/Runx1, and KLF4 mRNA expression levels in HT29-Cl.16E cells transfected with β-galactosidase–specific (control, white bars) or ZONAB/DbpA-specific (gray bars) siRNA for 72 hours. (C) Western immunoblotting detection of ZONAB/DbpA, AML1, and actin (loading control) protein expression levels, 72 hours after transfection of HT29-Cl.16E cells with ZONAB/DbpA 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) ALCIN blue staining (top) and Muc-2 flow cytometry (bottom) on HT29-Cl.16E control cells (NS) after transfection with β-galactosidase (control) or ZONAB/DbpA-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.22 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 AML1Δ/Δ 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 AML1Δ/Δ 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.

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 up-regulation 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...
References

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-Claux, 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

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<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>GAPDH</td>
<td>TGGCAAAGTGGATGCTCAT</td>
<td>AAGATGGTGATGGGCTTCCCG</td>
</tr>
<tr>
<td>SYMPK</td>
<td>TGCTGATGATCTTGAGGCTG</td>
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</tr>
<tr>
<td>MUC2</td>
<td>CATCTGTCCATTACAGACACG</td>
<td>GGGTGTGTGTTGTAGTGGTG</td>
</tr>
<tr>
<td>KLF4</td>
<td>CCCATCTCAAGGCACACC</td>
<td>GCATTACCCTGGCATCCTGG</td>
</tr>
<tr>
<td>RUNX1 (AML1)</td>
<td>TCCCTGAAACCACCTCCACTG</td>
<td>GATGAGTCATGCCTGTCCTG</td>
</tr>
<tr>
<td>ZONAB/DbpA</td>
<td>GCTGGGGAGGGAGGAGGA</td>
<td>CTGTTGGGATGTTGGATAGAC</td>
</tr>
</tbody>
</table>

B) Primer Sequences for ChIP Nested PCR

Promoter/Tx factor binding site

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX1/ZONAB</td>
<td></td>
</tr>
<tr>
<td>Outside pair</td>
<td></td>
</tr>
<tr>
<td>CCCCTCTTGAAAGTCTAC</td>
<td>TGACCTATGCTGGTTTCA</td>
</tr>
<tr>
<td>Inside pair</td>
<td></td>
</tr>
<tr>
<td>CGGCTATTTCTTGACACGC</td>
<td>GAAGTTTTCACACACCCAAA</td>
</tr>
<tr>
<td>KLF4/Runx1</td>
<td></td>
</tr>
<tr>
<td>Outside pair</td>
<td></td>
</tr>
<tr>
<td>ATAACTGGGCTCTTTTTCCAG</td>
<td>TCGTTGCTATGGACAGTCAAA</td>
</tr>
<tr>
<td>Inside pair</td>
<td></td>
</tr>
<tr>
<td>CGCGAACGGCGTATGCTA</td>
<td>GGGGGGAGGGGAAGGAG</td>
</tr>
</tbody>
</table>

C) Primer Sequences for AML1 Genomic DNA Recombination

Primer 5 (TIMER-FLP2): TAGGGAGTGCTGCTTGCTCT
Primer 6 (TIMER-FLP1): GCCGGGTCATATTAAGTCA
Primer 7 (AML-CRE1): CTCTGGGAACCCAGGGAGGAG

D) Primer Sequences for AML1/Runx1 cDNA Amplification

Runx1 exon 4/5-fw: AGCGACACCCATTTCACC
Runx1 exon 4/5-rev: GGAGATGGGACGGACAGAGTAG
mouse B2m-fw: GAGCCCAAGACCTGCTACTG
mouse B2m-rev: GCTATTTCTTTCTGCATGCAT

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

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TGGCAAAGTGGATGCTCAT</td>
<td>AAGATGGTGATGGGCTTCCCG</td>
</tr>
<tr>
<td>KLF4</td>
<td>CGGGAAGGGGAAGGACACT</td>
<td>GAGTTTTCAGCCACCAAGC</td>
</tr>
<tr>
<td>RUNX1 (AML1)</td>
<td>TCCCTGATACCACCTCCACTG</td>
<td>ATGACGGGTGACAGAGGAG</td>
</tr>
<tr>
<td>MUC2</td>
<td>CTGCTTTGGCCACCCCTCGT</td>
<td>TTCGGGATGCTGCTTCTT</td>
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</tbody>
</table>

July 2009 REGULATION OF DIFFERENTIATION BY SYMPLEKIN 164.e1
## F) Oligonucleotides Used for Electrophoretic Mobility Shift Assay

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Sense Oligo</th>
<th>Antisense Oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML1 POS</td>
<td>CAGTGGCTCATCGATTGTGGTTTCTTGTGC</td>
<td>CAGTGCAACAAGAAACCACAATCGATGAGCC</td>
</tr>
<tr>
<td>AML1-A</td>
<td>CAGTCGCCCGCTGACCCCACCAGTCTTCG</td>
<td>CAGTCGAAGACTGGTGCGGTCAGCGGGCG</td>
</tr>
<tr>
<td>AML1-B</td>
<td>CAGTCGCCGCCGACACCACCTGCGCGCCGCGC</td>
<td>CAGTGCCGCCGCGCCAGTGTGTCGCGCGCG</td>
</tr>
<tr>
<td>AML1 SCR</td>
<td>CAGTGCAAGATAAACTTAATGATCTTTCG</td>
<td>CAGTCAAAAGATCAATTAAGTTATCTTTC</td>
</tr>
</tbody>
</table>

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 40x 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).