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Control of Intestinal Promoter Activity of the Cellular Migratory Regulator Gene *ELMO3* by CDX2 and SP1

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ABSTRACT

An important aspect of the cellular differentiation in the intestine is the migration of epithelial cells from the crypt to the villus tip. As homeodomaine transcription factor CDX2 has been suggested to influence cell migration, we performed a genome-wide promoter analysis for CDX2 binding in the differentiated human intestinal cancer cell line Caco-2 in order to identify CDX2-regulated genes involved in cellular migration. The engulfment and cell motility 3 (*ELMO3*) gene was identified as a potential CDX2 target gene. ELMO3 is an essential upstream regulator of the GTP-binding protein RAC during cell migration. However, no information is available about the transcriptional regulation of the *ELMO3* gene. The aim of this study was to investigate the potential role of CDX2 in the regulation of the *ELMO3* promoter activity. Electrophoretic mobility shift assays showed that CDX2 bound to conserved CDX2 sequences and mutations of the CDX2-binding sites, significantly reduced the promoter activity. Reporter gene assays demonstrated that the region mediating *ELMO3* basal transcriptional activity to be located between −270 and −31 bp. Sequence analysis revealed no typical TATA-box, but four GC-rich sequences. In vitro analyses (electrophoretic mobility shift assays and promoter analyses) demonstrate that the SP1-binding sites are likely to play an important role in regulating the *ELMO3* promoter activity. Furthermore, we showed here that CDX2 and SP1 can activate the *ELMO3* promoter. Taken together, the present study reports the first characterization of the *ELMO3* promoter and suggests a significant role of CDX2 in the basal transcriptional regulation of the intestine-specific expression of *ELMO3*, possibly through interaction with SP1. J. Cell. Biochem. 109: 1118–1128, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ELMO3; CDX2; SP1; INTESTINE; MIGRATION

The intestinal epithelium is a self-renewing system undergoing continuous replacement from stem cells. These stem cells give rise to a layer of committed so-called transient-amplifying cells, which undergo a few cell divisions as they migrate toward the crypt openings, here the proliferation ceases and the cells can differentiate into three distinct cell types: absorptive enterocyte cells, mucus-secreting goblet cells, and enteroendocrine cells, and some cells migrate to the bottom of the crypt and differentiate into paneth cells [Cheng and Leblond, 1974]. Intestinal epithelial cells migrate along the basement membrane toward the small intestinal villus tip or colonic surface, whereupon enterocytes, goblet cells, and enteroendocrine cells lose the ability to adhere to the basement membrane and undergo programmed cell death as they are subsequently shed into the intestinal lumen, with a turnover rate of 3–5 days [Heath, 1996].

The continuous renewal of intestinal epithelium provides many unique challenges and is an example of a well-controlled cell death system which makes way for new differentiated and migrating cells without accumulation of cells and the risk of tumor development. Cell proliferation and differentiation are thus tightly controlled in the normal intestinal epithelium. Many of the molecular pathways regulating and controlling stem-cell renewal and differentiation in normal tissue have been identified, including *Wnt* signalling [Pinto et al., 2003], the bone morphogenic protein pathway [Hardwick et al., 2004], the phosphatidylinositol 3-kinase pathway [Vivanco and Sawyers, 2002], and the Notch pathway [Fre et al., 2005]. In addition, many of the transcription and growth factors that regulate intestinal cell proliferation and differentiation have been identified by studying the regulation of intestinal-specific genes like lactase-phlorizin hydrolase and sucrase-isomaltase. Such studies have

Abbreviations used: CDX2, caudal-related homeobox transcription factor 2; ChIP, chromatin immunoprecipitation; E2F4, E2F transcription factor 4; ELMO3, engulfment and cell motility 3; EMSA, electrophoretic mobility shift assay; HEPH, hephaestin; HA, hemagglutinin; IP, immunoprecipitation; PEPT1, H^+-coupled peptide transporter 1; qPCR, quantitative PCR.

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shown that the transcription of intestinal expressed genes can be activated in intestinal cell culture by transcription factors like HNF-1α/β, HNF-4α, GATA factors, CDX2, and SP1 [Boukamel and Freund, 1994; Troelsen et al., 1997; Boudreau et al., 2001; Fang et al., 2001; Stegmann et al., 2006].

The caudal-related homeobox transcription factor 2 (CDX2) plays a fundamental role in the mammalian embryonic development, as inactivation of both *Cdx2* alleles (*Cdx2*$^{-/-}$) leads to early lethality in mouse embryos [Chawengsaksophak et al., 2004]. Furthermore, a recent study with conditional *Cdx2* knockout mice shows that Cdx2 is a master regulator in the posterior endoderm and essential for the establishment of intestinal identity [Gao et al., 2009]. In adult mammals, the CDX2 expression becomes selectively expressed in the crypt–villus epithelium of the small intestine and colon [Silberg et al., 2000], but is active in differentiating enterocytes [Rings et al., 2001]. Besides its homeotic role in gut development and maintenance, CDX2 may also function as an important tumor suppressor in the colon. Thus, *Cdx2*$^{+/-}$ heterozygote mice developed six times more polyp-like lesions than the wildtype mice [Chawengsaksophak et al., 1997], and *Cdx2*$^{+/-}$ mice treated with a carcinogen, azoxymethane, had an increased risk for colon cancer development [Bonhomme et al., 2003]. Immunohistochemistry analysis using a CDX2-antibody showed a reduced expression of CDX2 in most of the investigated human colon cancer cases [Kaimaktchiev et al., 2004; Choi et al., 2006]. It is not clear what role CDX2 has in the migration process of cells and metastatic spreading of colon cancer [Rao et al., 1999; Brabletz et al., 2004; Gross et al., 2008]. While some studies have shown that over-expression of CDX2 in IEC-6 cells increased cell migration four times after wounding compared to the non-transfected IEC-6 cells [Rao et al., 1999], other recent studies indicate that expression of CDX2 in human colon cancer cell lines retarded wound repair and reduced migration [Gross et al., 2008]. All this taken together indicates that CDX2 is a tumor suppressor, that reduction in its expression increases the risk for adenocarcinomas in colon, and that CDX2 has a critical function in intestinal development, differentiation, and possibly in migration [Uesaka et al., 2004].

In the present study, we performed a genome-wide chromatin immunoprecipitation (ChIP)–chip promoter analysis for CDX2 binding to chromatin in differentiated Caco-2 cells. This revealed the human *ELMO3* gene as a potential CDX2 target. Thus, we examined the regulation of the *ELMO3* promoter activity in vitro and demonstrated that CDX2 together with SP1 play important roles in regulating the *ELMO3* promoter activity in Caco-2 cells. These data are the first to demonstrate that CDX2 regulates the expression of a migratory gene, thereby signifying the importance of CDX2 for cell differentiation in the intestinal epithelium.

MATERIALS AND METHODS

CLONING OF THE *ELMO3* PROMOTER

Cloning of the 1,408 bp flanking region upstream of the *ELMO3* gene (from position −1438 to −31) into a modified firefly luciferase reporter vector, pGL4.10 (Promega), was performed using Gateway® Recombination Cloning Technology (Invitrogen). The Gateway Reading Frame Cassette C.1 was cloned into the *Eco*RV site of the pGL4.10 plasmid producing the Gateway destination vector pGL4-DEST. The *ELMO3* promoter was amplified from human genomic DNA (Roche Diagnostics) with *ELMO3* promoter primers (hELMO3 F and hELMO3 R (Table I)) and cloned into the pCR®8/GW/TOPO® vector (Invitrogen), resulting in the pCR8/GW/TOPO-ELMO3 construct. Finally, a recombination reaction was performed between the pCR8/GW/TOPO-ELMO3 and the pGL4-DEST vector to transfer the *ELMO3* promoter to the reporter plasmid according to the manufacturer's manual, yielding the *ELMO3* promoter/reporter gene plasmid pGL4-ELMO3−1438/−31.

Site-directed mutations in the putative CDX2- and SP1-binding sites in the *ELMO3* promoter were introduced in a two-step PCR-based approach, with primers listed in Table I. A 5′-deleted promoter construct pGL4-ELMO3−270/−31 was generated by digesting pGL4-ELMO3−1438/−31mutCDX2-B with *Hin*dIII, and the resulting fragment was gel-purified and subcloned into pGL4.10. All the

TABLE I. List of Primers Used for Genomic DNA Amplification, qPCR, RT-PCR, Mutagenesis[a] and EMSA

Name	Purpose	Forward primer	Reverse primer
hELMO3	Cloning	GACCTTGTTGTGGCGCTTAT	TAGTTCCGGGTTGGAGTCTG
HEPH	ChIP	AGCAGAGGCCTTATCCCTTC	GCTGAGATCCAAGTCCAAGC
ELMO3	ChIP	CATTTAGAGCCATTTGCAGAGA	AGCTAGCCTTAGGCATGGTTT
ELMO3	RT-PCR	GTACAGCCGGTTTGTGTTGGA	AACATGGGTGAGAAGTCCTGG
HEPH	RT-PCR	TGTGGCCTCAACTGACTGATG	GCTGGTTCGTGATGACATTTCT
CDX2	RT-PCR	CGGAGGAAAGCCGAGCTAGCC	GCGGAGGCGGCTGTGGTG
wtCDX2-A	EMSA	ATTCCTATATAAAGATTATTTTTAT	TATAAAAATAATCTTTATATAGGAA
wtCDX2-B	EMSA	TTCAAGTGAATAAAAACCATGCCT	TAGGCATGGTTTTTATTCACTTGA
mutCDX2-A	Mut/EMSA	ACGAATGGATTCCTAGGATCCGATTATTTTTATACT	AAGTATAAAAATAATCGGATCCTAGGAATCCATTCG
mutCDX2-B	Mut/EMSA	ACAGTGTTCAAGTGAAAGCTTACCATGCCTAAGGCT	TAGCCTTAGGCATGGTAAGCTTTCACTTGAACACTG
Sp1-consensus	EMSA	TATTCGATCGGGGCGGGGCGAGC	TGCTCGCCCCGCCCCGATCGAAT
wtSP1-A	EMSA	CTGGAACTAGGGGTGGGGCGGCCTCC	CGGAGGCCGCCCCACCCCTAGTTCCA
wtSP1-B	EMSA	GCCTGCCCTCCCCGCCCCCACCTGCC	CGGCAGGTGGGGGCGGGGAGGGCAGG
wtSP1-C	EMSA	ACCTTGGTGTGGGGCGGGGCCGCCTC	CGAGGCGGCCCCGCCCCACACCAAGG
wtSP1-D	EMSA	GCCTCGGCGGGGCGGGGCAGGCGGAG	GCTCCGCCTGCCCCGCCCCGCCGAGG
mutSP1-A	Mut/EMSA	CTTGCTGGAACTAGAAGTGAAGCGGCCTCCGGCAG	CTGCCGGAGGCCGCTTCACTTCTAGTTCCAGCAAG
mutSP1-B	Mut/EMSA	GGCAGCCTGCCCTCAACGCAACCACCTGCCGCACC	GGTGCGGCAGGTGGTTGCGTTGAGGGCAGGCTGCC
mutSP1-C	Mut/EMSA	CGCACCTTGGTGTGAAGCGAAGCCGCCTCGGCGGG	CCCGCCGAGGCGGCTTCGCTTCACACCAAGGTGCG
mutSP1-D	Mut/EMSA	GGGCCGCCTCGGCGAAGCGAAGCAGGCGGAGCCGT	ACGGCTCCGCCTGCTTCGCTTCGCCGAGGCGGCCC

[a]Mutations introduced are underlined.

constructed plasmids were sequenced and analyzed on an ABI 3100 sequencer (Applied Biosystems).

CELL CULTURING AND TRANSFECTION

Human colon carcinoma Caco-2 cell line, confluent HT29 cells, and HeLa cells were used in this study. HT29 cells were grown in the same conditions as previously described by Olsen et al. [2003]. Caco-2 cells were grown in DMEM containing 10% fetal calf serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at a density of 5×10^4 cells/ml of medium and grown in T175 (175 cm^2, Nunc) flasks at 37°C, in a 5% CO_2 atmosphere and 90% humidity. Caco-2 cells were transiently transfected using ExGen500 (Fermentas). The total of 70–80% confluence Caco-2 cells were plated into 24-well plates (Costar) at a density of 5×10^4 cells/well and transfected the following day with 5 μl 5.47 mM ExGen500. In each transfection experiment, four replications were done, and each transfection was carried out twice. For co-transfections, pCMV-Cdx3 (a hamster CDX2 expressing plasmid kindly provided by Dr. Michael German [German et al., 1992]), pCMV-SP1, empty pcDNA3.1 (+) expression vector, or a combination of these expression vectors were added together with 0.1 μg pCMV-lacZ plasmid (as internal transfection control) and normalized to a total amount of 0.3 μg DNA with pBluescript SK^+ plasmid. The cells were harvested, and 2 days after transfection the luciferase and β-galactosidase activities were analyzed using the Dual Light® system (Tropix; Perkin Elmer). Each luciferase activity was normalized to β-galactosidase activity presented as relative luciferase activity, except for the CMV–SP1 over-expression, which significantly stimulated the β-galactosidase expression.

ELECTROPHORETIC MOBILITY SHIFT AND SUPERSHIFT ASSAYS

The probes shown in Table I, for the electrophoretic mobility shift assays (EMSAs) were designed to cover putative CDX2- or SP1-binding sites, predicted by an in silico analysis (Match™ from TRANSFAC® Professional 12.1 database [Matys et al., 2003]). The probes were prepared by annealing complementary sense and antisense oligonucleotides, followed by 5′-end-labeling with [γ-^{32}P]ATP (Perkin Elmer) and T4 polynucleotide kinase (Fermentas) and purified through a Microspin G-25 column (GE Healthcare). The binding mixtures were performed using 2–5 μg differentiated Caco-2 or HeLa nuclear extracts [prepared as previously described by Troelsen et al. [1997]] in 3–5 μl dialysis buffer (25 mM Hepes pH 7.6, 40 nM KCl, 0.1 mM EDTA ,and 10% glycerol), 10 μl gel-shift buffer (25 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 60 mM KCl, 0.5 mM EDTA, 5% Ficoll 400, 2.5% glycerol, 1 mM DTT, 1 μl/ml protease inhibitor cocktail (Sigma Aldrich), and 5 μg BSA) and in the presence of 1 μg poly-(dI:dC) with 0.1 μg sonicated salmon DNA. After preincubation for 20 min on ice, labeled probes (25 fmol) were added and the binding mixtures were incubated for a further 20 min. For the competition assays, a 100-fold (2.5 pmol) excess of unlabeled probes was added to the binding mixtures, before the addition of nuclear extracts. For supershift assays, mouse monoclonal CDX2-antibody against human CDX2 [α-CDX2 (MU392A-UC), BioGenex], rabbit polyclonal Cdx2-antibody against hamster Cdx2 (α-Cdx3, a gift from Dr. Michael German), rabbit polyclonal SP1-antibody against human SP1 [α-SP1 (sc-59); Santa Cruz Biotechnology], or rabbit polyclonal SP3-antibody against human SP3 [α-SP3 (sc-644); Santa Cruz Biotechnology] were added to the binding mixtures and incubated for a further 20 min. The protein/DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel. Gels were dried on a Savant Slab Gel Dryer SGD4050 and exposed on a phosphor screen. The screens were scanned using a Storm 829 scanner from Molecular Dynamics, and the protein/DNA complexes were analyzed by the Image-Quant Software version 3.5.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

Differentiated Caco-2 cells grown on a 245 mm × 245 mm tissue culture dish (Nunc) for 10 days after confluence were cross-linked with a formaldehyde solution (11% formaldehyde, 0.1 M NaCl, 1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, and 50 mM HEPES pH 8.0) added to the culture media to a final concentration of 1% at room temperature for 30 min. Fixed cells were washed twice with ice-cold PBS, scraped off the plate, washed with ice-cold PBS containing protease inhibitors, and resuspended in 6 ml SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.0, and protease inhibitors). The cells were then sonicated using a Branson Digital Sonifier 450 with repeated cycles of (level: 50%; sonication time: 24 × 15 s; resting time between each pulse: 30 s). The samples were centrifuged at 4°C and 10,000*g* for 15 min, the supernatants were collected and split into 300 μl aliquots, diluted with 1.2 ml ChIP buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, and protease inhibitors), and precleared for 1 h at 4°C with a protein A/G sepharose beads slurry [25 μl TE buffer, 12.5 μl protein A, and 12.5 μl protein G sepharose beads (GE Healthcare), 0.2 mg/ml sonicated salmon DNA, and 0.5 mg/ml lipid-free BSA] to reduce the non-specific background. After mixing, the samples were spun at 20,000*g* for 30 min at 4°C, and the beads were carefully discarded. An aliquot of 15 μl corresponding to 1% of each chromatin supernatant was reserved as input controls. The rest of the chromatin supernatants were immunoprecipitated (IP) overnight at 4°C on rotation with monoclonal antibodies specific for either human CDX2 [65.2 μg/ml α-CDX2 (MU392A-UC), BioGenex] or an influenza hemagglutinin (HA) protein [200 μg/ml α-HA (sc-805), Santa Cruz Biotechnology] used as a negative control. Immunocomplexes were recovered by the addition of protein A/G sepharose beads and further incubated for 2 h at 4°C on rotation. The beads were washed successively two times with 1 ml of low-salt washing buffer 1 (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, and 20 mM Tris–HCl pH 8.0), followed by two washes with high-salt washing buffer 2 (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 500 mM NaCl, 1 mM EGTA, 2 mM EDTA, and 20 mM Tris–HCl pH 8.0), one wash with washing buffer 3 (0.25 M LiCl, 0.5% sodium deoxycholate, 0.5% nonidet P-40, 0.5 mM EGTA, 1 mM EDTA, and 10 mM Tris–HCl pH 8.0), and two final washes with TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA). Immunocomplexes were eluted with 300 μl fresh elution buffer (1% SDS, 0.1 M $NaHCO_3$, and 200 mM NaCl) and incubated at room temperature for 30 min with rotation. After centrifugation, the cross-links were reversed by incubation overnight on a heat block at 65°C. The samples were further incubated for 30 min at 37°C with 240 μl TE buffer, 1.5 μl of 20 mg/

ml glycogen, and 10 μl of 10 mg/ml RNase A. Then, the chromatin-associated proteins were digested with 8 μl of 12.5 mg/ml proteinase K at 55°C for 2 h. The DNA was finally recovered by buffered phenol–chloroform extraction (pH 7.9; Invitrogen), and ethanol precipitated, and resuspended in 200 μl Milli-Q H_2O.

qPCR AND RT-PCR ANALYSIS

The promoter sequences of *ELMO3* and Hephaestin (*HEPH)* genes were defined as the upstream region of sequences from GenBank database [refseq gene accession No. NM_024712 (*ELMO3*) and NM_138737 (*HEPH*)]. We used an intron in the *IgG* gene (IgG) as a negative control for CDX2 binding as recently described by Boyd et al. [2009]. Promoter-specific primers listed in Table I were designed using Primer3 software [Rozen and Skaletsky, 2000] for quantitative PCR (qPCR) analysis. qPCR was performed with a LightCycler® (Roche Diagnostics) using SYBR® Premix Ex Taq™ kit (Takara) with 5 μl purified IP DNA or input genomic DNA as a template. Quantification of the ChIP DNA was done using the method described by Frank et al. [2001]. Total RNA was extracted by using E.Z.N.A total RNA kit (Omega BioTek) as previously described by Schjoldager et al. [2008]. The list of primers used in the semi-quantitative RT-PCR is given in Table I, and the PCR products were run in a 3% agarose gel. The thermocycling parameters used in the PCR were as follows: one cycle for 3 min at 94°C; 30 cycles for 45 s at 94°C, 45 s at 58°C, 1 min at 72°C; and one terminal cycle for 5 min at 72°C.

ChIP–Chip ANALYSIS

The CDX2 ChIP purified DNA and genomic input DNA were amplified as described by [Odom et al., 2004]. The amplified ChIP DNA was labeled and hybridized to a 1.5 kb promoter array containing probes covering the region from −1,350 to +150 of 24.275 human genes by NimbleGen Systems Inc. (see http://www.nimblegen.com/products/chip/index.html for a description of the promoter array). The data from the three CDX2 promoter array analyses were extracted according to standard operating procedures by NimbleGen Systems Inc. The enrichment of CDX2 IP DNA was calculated as $\log_2$ ratio between the CDX2 IP DNA and the genomic input DNA.

RESULTS AND DISCUSSION

IDENTIFICATION OF THE ELMO3 GENE AS A NOVEL CDX2 TARGET

In order to identify potential CDX2 target genes, a combined ChIP technique with DNA microarray analysis (ChIP–chip) was used. First, a whole genome promoter analysis was performed with chromatin isolated from differentiated Caco-2 cells. Using CDX2 IP DNA, we interrogated a promoter array containing tiled oligonucleotide probes from almost 25,000 human promoters. The CDX2 ChIP–chip data was matched with previously collected ChIP–chip data from Caco-2 using antibody against lysine 9 (K9) acetylated histone H3 [Boyd et al., 2009]. Acetylation of histone H3 at K9 in promoter indicates an open chromatin structure available for transcription [Nishida et al., 2006]. Interestingly, the ChIP–chip study revealed that the promoter of the *ELMO3* gene (involved in cell migration) is bound by CDX2. From the ChIP–chip data, clear signals within the probes located at positions −48, −208, −438, and −578 in the *ELMO3* promoter were retrieved (Fig. 1A). The ChIP–chip analysis of acetylated histone H3 indicated that the chromatin of the *ELMO3* gene is acetylated in the transcription initiation site of *ELMO3* and partly overlapping the CDX2 signal (Fig. 1A). This indicates that the promoter has an open chromatin structure. Based on these results, we consider *ELMO3* as a possible CDX2 target gene in the differentiated cancer cell line Caco-2.

The ELMO family of proteins is a group of evolutionarily conserved orthologs of the *Caenorhabditis elegans* ced-12 protein. Three orthologs, ELMO1, ELMO2, and ELMO3, have been identified in mammals [Gumienny et al., 2001]. ELMO1 is the most studied to date, but from homology and similarity studies it is expected that ELMO2 and ELMO3 possess the same functions. Studies suggest that ELMO proteins are scaffold proteins with no obvious catalytic domain [Gumienny et al., 2001]. The N-terminus of the ELMO proteins has a region which binds to the small GTP-binding protein RHOG [Katoh and Negishi, 2003] and to the ERM protein family [Grimsley et al., 2006], which has been implicated in processes in embryonic development, formation of microvilli, cell motility, and formation of membrane ruffles. A central domain comprises an ELMO domain, specific to ELMO proteins [Bowzard et al., 2007]. Finally, the C-terminal consists of an atypical pleckstrin homology-like domain [Zhou et al., 2001] and a complex proline-rich Src homology-3 binding region, interacting with a 180 kDa protein called DOCK180 [Gumienny et al., 2001]. Structural analyses have revealed that ELMO proteins form a complex with DOCK180 to function as a two-part guanine nucleotide exchange factor for RAC activation [Komander et al., 2008] at the cell membrane [Katoh and Negishi, 2003] during engulfment and cell migration processes [Grimsley et al., 2004]. Although ELMO proteins stimulate cell migration in normal mammalian cells and cancer cells, it has not been investigated whether these proteins play a critical role in the renewing processes along the crypt–villus axis in intestines.

To verify the ChIP–chip signal of CDX2 analysis of the *ELMO3*, a qPCR analysis was performed on CDX2 ChIP purified DNA from differentiated Caco-2 cells using *ELMO3* promoter-specific primers flanking the potential CDX2-binding region (Fig. 1A,B). *HEPH* promoter was used as a positive control for CDX2 binding, as CDX2 plays a key role in regulating the expression of the iron transport protein HEPH in intestinal and colonic epithelium [Hinoi et al., 2005]. The enrichments are shown in Figure 1B and reveal significant ($P < 0.05$) enrichment of CDX2 ChIP DNA for both *ELMO3* and *HEPH* but not for *IgG*-control, compared to negative HA–ChIP control samples.

Next, we performed a semi-quantitative RT-PCR analysis using gene-specific primers listed in Table I to examine the expression levels of *ELMO3*, *HEPH*, and *CDX2*. As shown in Figure 1C, *ELMO3*, *HEPH*, and *CDX2* were expressed both in Caco-2 and also in another intestinal cancer cell line HT29, as confluent HT29 cells have been shown to have endogenous levels of CDX2 [Modica et al., 2009]. We demonstrate that *ELMO3* has almost the same expression levels in both cell lines, whereas *HEPH* and *CDX2* are highly expressed in Caco-2 cells compared to HT29 cells. These data indicate that *ELMO3* are expressed in Caco-2 and HT29 cells and it is therefore likely that *ELMO3* is a potential migratory regulator in intestinal cells.

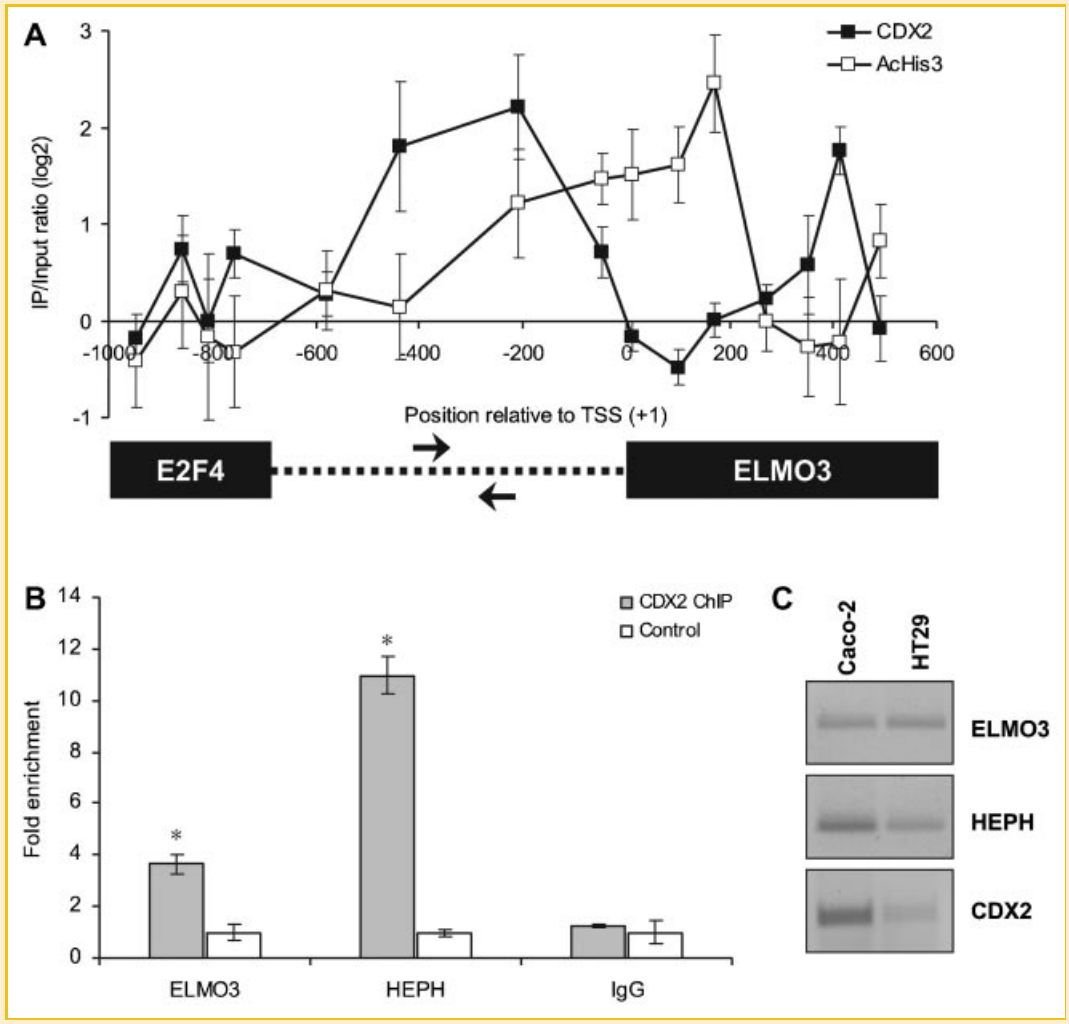

Fig. 1. Identification of *ELMO3* as a novel CDX2 target gene. CDX2 ChIP–chip analyses on differentiated Caco-2 cells were performed in triplicates using 1.5 kb human whole genome promoter arrays from NimbleGen/Roche. A: Representation of the hybridization of ChIP-enriched DNA to the genomic locus for *ELMO3* is presented as the $\log_2$ ratios of signals for tiled probes for CDX2 (■) and lysine 9 acetylated histone H3 (□) enriched IP DNA divided by non-enriched input DNA and represent the mean ± SD (N = 3). Parts of the *ELMO3* and E2F transcription factor 4 (*E2F4*) gene coding regions covered by the probes are shown by boxes in the bottom. The arrows indicate primers used in the real-time qPCR. B: Gene-specific qPCR analysis with primers spanning the CDX2-binding regions in the *ELMO3*, positive control *HEPH*, and an IgG intron as a negative control. Equal amounts of DNA from input, CDX2-antibody enriched samples, or negative controls using hemagglutinin (HA)-antibody in the ChIP were used. Enrichments are presented as fold enrichments compared to HA-control (control) and represent the mean ± SD (N = 3). $^*P < 0.05$, significantly different from the negative HA-control. C: Semi-quantitative RT-PCR analysis of *ELMO3*, *HEPH*, and *CDX2* expression in confluent Caco-2 and HT29 cells.

REGULATION OF THE ELMO3 PROMOTER ACTIVITY BY CDX2

The examination for putative CDX2 DNA-binding sites upstream of the *ELMO3* gene was investigated using the Match™ tool from the TRANSFAC® database [Matys et al., 2003]. The Match™ analysis predicted two conserved putative CDX2-binding sites as shown in Figure 2A between −337 and −333 (ATAAA; CDX2-A) and −271 to −267 (ATAAA; CDX2-B). As shown in Figure 2B, the sequence (−360 to −250) is highly conserved among different mammalian species.

To examine the interaction between the putative CDX2 sites of the human *ELMO3* promoter and the CDX2 homeodomain protein, EMSAs and supershift assays were performed. The CDX2-A probe, derived from the human *ELMO3* promoter, formed two protein/DNA complexes with the Caco-2 nuclear extracts (complexes I and II, Fig. 3A, lane 1). The formation of both complexes was competed efficiently by addition of 100-fold excess unlabeled CDX2-A oligonucleotides (lane 2), but complex II was also competed with unlabeled excess mutant-type oligonucleotides (lane 3). This observation could possibly be explained by the presence of a second CDX2 site in the CDX2-A probe which was not mutated (Table I). The non-specific oligonucleotides had no significant competitive effects on the complex formations (lane 4) demonstrating a specific interaction in complexes I and II. Addition of CDX2-antibody did not show a clear supershift of either complex I or II (lane 5); however, we would expect that a CDX2/DNA complex would migrate as complex II (compared Fig. 3A to Fig. 3B). In that case, a supershifted band would co-migrate with complex I (lane 5); however, we were not able to see a depletion of complex II. We have previously shown that HeLa cells have no endogenous expression of

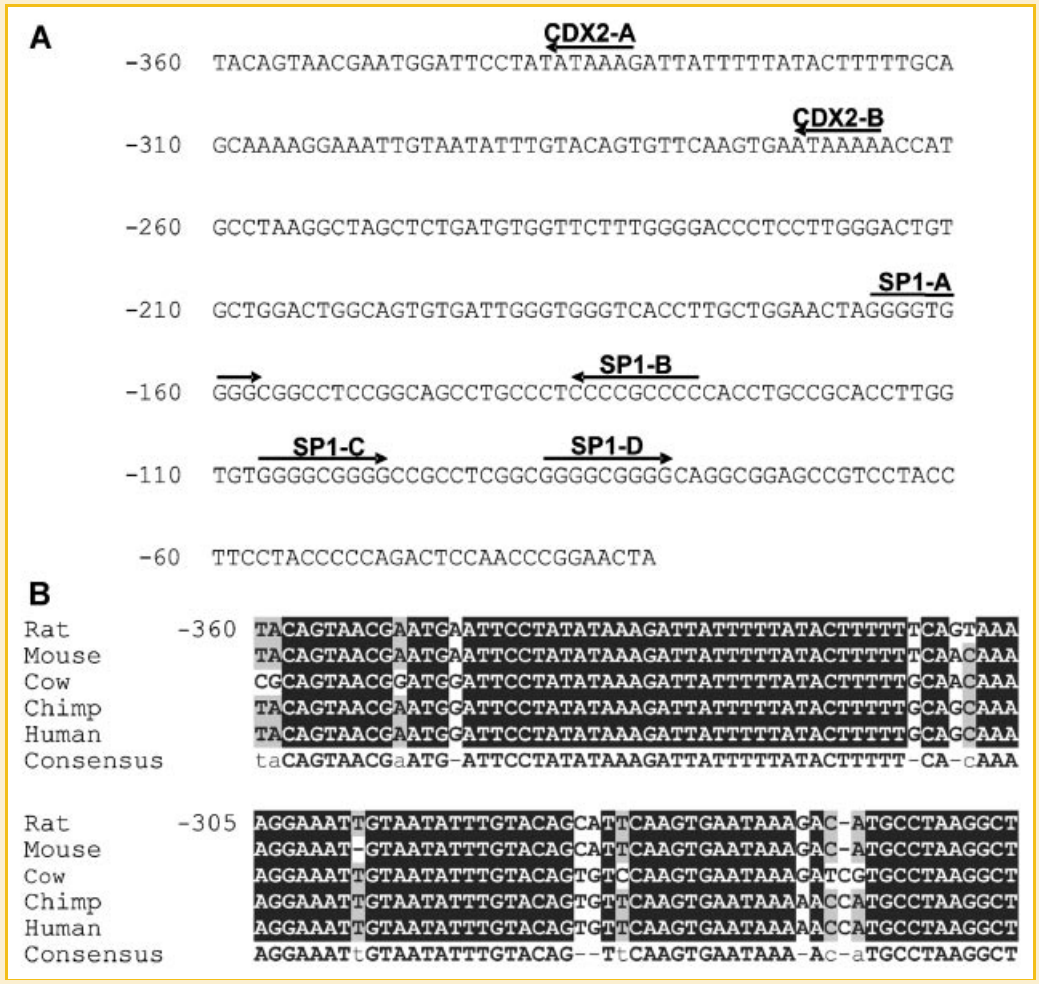

Fig. 2. CDX2- and SP1-binding sites in the *ELMO3* promoter. A: The nucleotide sequence of the *ELMO3* gene promoter from −360 to −30 bp relative to transcription start site. CDX2 and SP1 transcription factor binding sites were identified using TRANSFAC® Professional 12.1 database. Potential CDX2- and putative SP1-binding sites are indicated by arrows. B: Conservation of the *ELMO3* promoter region in mammals. Alignment of the *ELMO3* promoter sequence from −360 to −250 bp with sequences from rat, mouse, cow, and chimpanzee defined as the upstream region of sequences from GenBank database (The GenBank refseq gene accession No. for the *ELMO3* nucleotide sequence of Human NM_024712; Mouse NM_172760; Rat NM_001030028; Cow NM_001102091; and Chimpanzee NM_024712).

CDX2 [Troelsen et al., 1997], therefore, we performed an EMSA using Cdx2-transfected HeLa cell extracts. The CDX2-A probe demonstrated a supershift of complex II with the Cdx2-antibody, which can be seen clearly as the transfected HeLa cells do not contain complex I (Fig. 3C). Based on these results, we suggest complex II to be CDX2/DNA, whereas the nature of complex I is unknown (Fig. 3A). EMSAs with the CDX2-B probe resulted in a strong specific band (Fig. 3B, lane 1), as the complex was competed by the unlabeled CDX2-B oligonucleotides (lane 2) but not with the mutant CDX2-B (lane 3) or with the non-specific oligonucleotides (lane 4). Furthermore, the complex was supershifted with CDX2-antibody (lane 5), demonstrating the presence of CDX2 in the complex. The results were confirmed using Cdx2-transfected HeLa extracts (Fig. 3D). Taken together, these results suggest that CDX2 interacts with the identified CDX2-consensus sequences in the *ELMO3* promoter.

To determine the functional importance of the CDX2-binding sites for the *ELMO3* promoter activity, we cloned the 5′-flanking sequence of *ELMO3* (−1438 to −31) in front of a luciferase reporter gene (pGL4-ELMO3−1438/−31) and it was transfected into Caco-2 cells. Mutations of the CDX2-A or CDX2-B sites (pGL4-ELMO3−1438/−31mutCDX2-A and pGL4-ELMO−1438/−31mutCDX2-B, respectively) were also analyzed by transfection of Caco-2 cells, and the relative luciferase activities were compared to that of the wildtype. The *ELMO3* promoter is able to activate reporter gene expression efficiently as shown in Figure 4. A mutation introduced to the CDX2-B site did not affect the activity significantly. However, the promoter activity was reduced by almost one-third by the CDX2-A mutation, and a double mutation of both CDX2-A and CDX2-B (pGL4-ELMO3−1438/−31mutCDX2-A+B) reduced the activity of the *ELMO3* promoter significantly to about 45% (Fig. 4). Although the clear CDX2/DNA band was observed (Fig. 3B), a mutation only in the CDX2-B site did not significantly affect the activity (Fig. 4). A truncated promoter construct (pGL4-ELMO3−270/−31) containing 240 bp from the 3'-end of the promoter with deletions of all putative CDX2-binding sites had

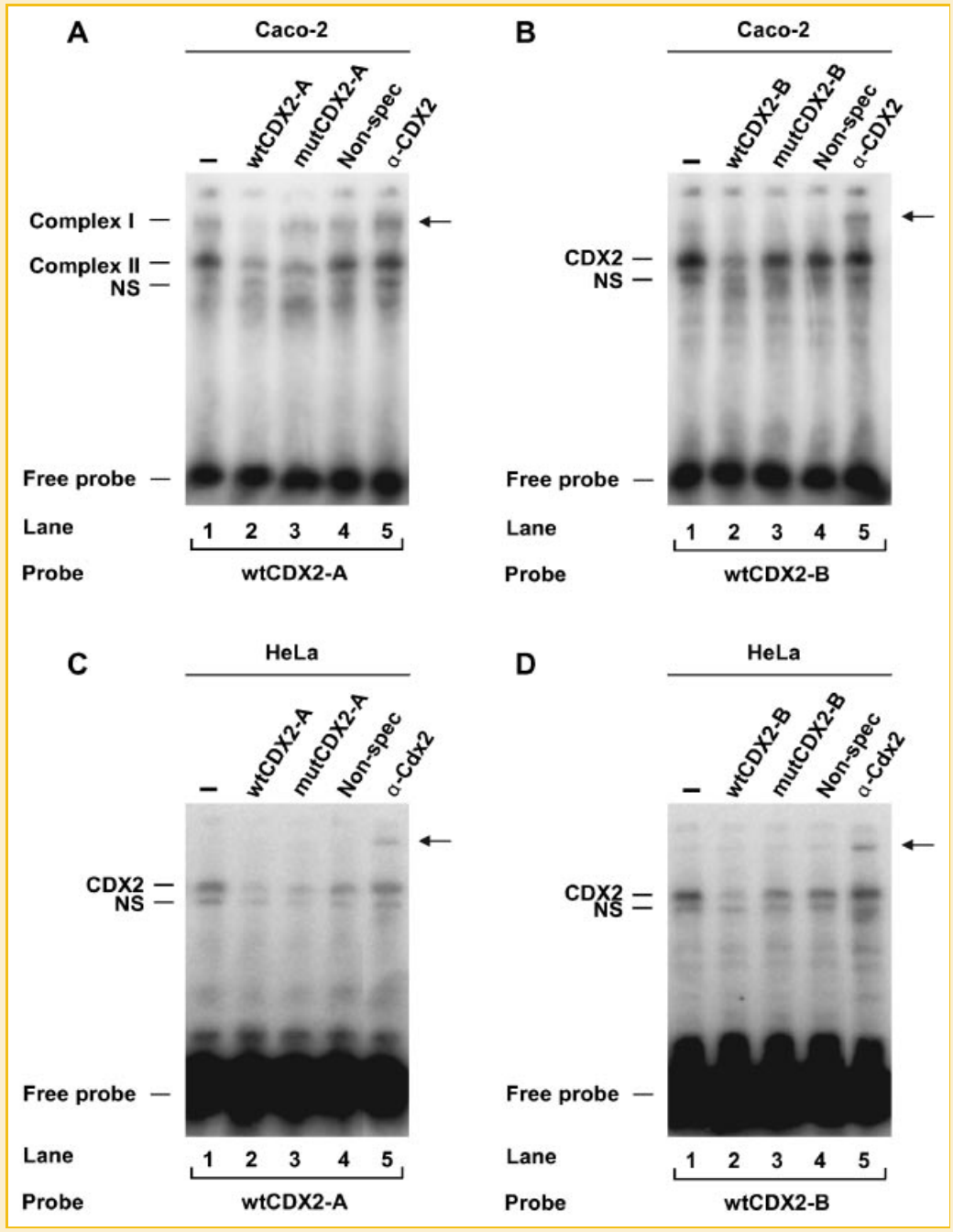

Fig. 3. EMSA for the *ELMO3* promoter. Nuclear extracts from Caco-2 cells incubated with the [γ^{32}P]ATP labeled wtCDX2-A (A), or wtCDX2-B probes derived from the human *ELMO3* promoter (B), and nuclear extracts from HeLa cells transfected with hamster Cdx2 were incubated with radioactive-labeled wtCDX2-A (C) or wtCDX2-B probes (D). Incubation of labeled probes with nuclear extracts resulted in shifted protein/DNA complex bands (lane 1). The competition assays were performed by adding a 100-fold excess of unlabeled (lane 2), mutant (lane 3), and non-specific oligonucleotides (lane 4). Anti-human CDX2 or anti-hamster Cdx2 (α-CDX2 or α-Cdx2) antibody were used for the supershift assays (lane 5). The addition of α-CDX2 or α-Cdx2 resulted in supershifted complexes indicated by arrows. NS, non-specific.

significant activity, suggesting that the region from −270 and −31 bp contains the sequence of nucleotides necessary for basal transcription of the *ELMO3* gene (Fig. 4). This demonstrated that the CDX2-binding sites in the *ELMO3* promoter are functionally involved in an activation of the *ELMO3* promoter activity. Also, a recent study by Gao et al. [2009] demonstrated that in a conditional *Cdx2* knockout mouse, the expression level of *ELMO3* in the small intestine is reduced by one-third, compared to the wild-type mouse. Taken together, these results demonstrate that lacking expression of CDX2 leads to a reduced *ELMO3* promoter activity, and these data furthermore indicate that ELMO3 is a CDX2 target gene in human and mouse.

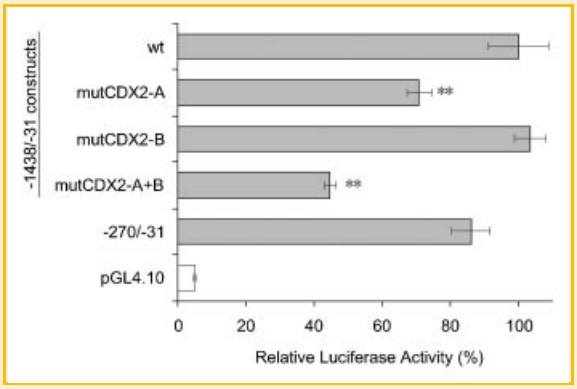

Fig. 4. Functional analysis of the *ELMO3* promoter. Mutated *ELMO3* promoter luciferase constructs pGL4-ELMO3−1438/−31mutCDX2-A, pGL4-ELMO3−1438/−31mutCDX2-B, and pGL4-ELMO3−1438/−31mutCDX2-A+B were generated through the introduction of a 5–6 nt substitution of CDX2-consensus binding sites using site-directed mutagenesis. Wild-type pGL4-ELMO3−1438/−31, mutated, or truncated (pGL4-ELMO3−270/−31) *ELMO3* promoter constructs (200 ng) were transiently transfected into Caco-2 cells. Firefly luciferase activities were normalized to β-galactosidase activity. The luciferase activities represent the mean ± SD (N = 4) in percentage compared to the wildtype (control). $^{**}P < 0.001$, significantly different from the wildtype.

SP1 AND SP3 BIND TO THE −270 TO −31 BP REGION OF THE ELMO3 PROMOTER

As the −270/−31 region of the *ELMO3* promoter had a relatively high promoter activity, we analyzed the −270/−31 region for potential transcription factor binding sites and found that the region contains several GC-rich sequences [Fig. 2A; located between −166 and −158 (GGGGTGGGG; SP1-A), −136 to −128 (CCCCGCCCC; SP1-B), −107 to −99 (GGGGCGGGG; SP1-C), and −88 to −80 (GGGGCGGGG; SP1-D)]. However, it contains no canonical TATA-box. SP family proteins play a complex role in the regulation of many genes, including structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors, and signal receptors (reviewed in Wierstra, 2008). SP1, SP3, and SP4 are well-characterized transcription factors that bind to GC-, CT-, and GT-boxes, with identical affinities [Hagen et al., 1994]. As many TATA-less promoters contain one or more SP1-binding sites to recruit the general transcription factors [Shimakura et al., 2005; Kajiwara et al., 2007], we investigated whether SP proteins interact with the GC-rich sequences in the *ELMO3* promoter.

To determine whether SP proteins binds to these putative GC-rich sequences within the −270/−31 region of the *ELMO3* promoter, EMSA and supershift assays were performed with the SP1-A, SP1-B, SP1-C, and the SP1-D double strand probes (Fig. 5A,B). All four probes gave three shifted protein/DNA complexes after incubating with Caco-2 nuclear extracts (lane 1). Complex formation was competed by the addition of 100-fold excess of unlabeled probes (lane 2) or SP1-consensus oligonucleotides (lanes 3), but not by mutant oligonucleotides in which SP1-binding sites were mutated (lane 4). Addition of antibodies specific for SP1 or SP3 resulted in supershifted bands as indicated by arrows (lanes 5 and 6). In agreement with other studies which have shown the migration patterns for SP1/DNA and SP3/DNA complexes [Abdelrahim et al., 2004; Abdelrahim and Safe, 2005], the present results together demonstrate that the four GC-rich sequences of the *ELMO3* promoter are SP1- and SP3-binding sites. However, for the SP1-A probe, the lower band is partly competed (lanes 2 and 3) due to the formation of non-specific complexes co-migrating with the lower band of the specific SP3/DNA complexes. With the SP1-B probe we also observed an unknown complex (lane 3) which was efficiently competed by the unlabeled SP1-B oligonucleotides, but with the SP1-consensus oligonucleotides it was only partly competed (lane 3), and did not efficiently supershift with SP3-antibody (lane 6), therefore, we suggest that another nuclear factor in addition to SP1 and SP3 bind to SP1-B. Abdelrahim et al. also characterized and demonstrated that SP1 and SP4 complexes co-migrate in EMSA [Abdelrahim et al., 2004; Abdelrahim and Safe, 2005]. Since SP4 is expressed in colon cancer cells [Abdelrahim and Safe, 2005; Chintharlapalli et al., 2009] this may explain why the SP1-antibody does not supershifting all of the upper protein/DNA complex (lane 5). Taken together, based on the presented EMSAs and supershift assays, we can conclude that the upper band is formed from SP1 binding, whereas the two lower bands results from SP3 binding. However, it is likely that the upper band also includes SP4 complexes.

An SP1-consensus oligonucleotide was used to investigate the ability of SP1-A, -B, -C, and -D to compete for binding of SP-proteins. An EMSA with the SP1-consensus oligonucleotide resulted in three protein/DNA complex bands (Fig. 5C, lane 1). SP1-consensus oligonucleotides competed the protein/DNA complex formations (lane 6) as well as excess of each of the four SP1 ELMO3 sequences (SP1-A, SP1-B, SP1-C, or SP1-D oligonucleotides; lanes 2–5), whereas addition of excess unlabeled non-specific oligonucleotides had no effect on the band intensities (lane 7). Co-incubation with SP1- and SP3-antibody gave supershifted bands (lanes 8 and 9). We did not see an efficiently competition of the lower SP3 band (lanes 2–6), possible caused by an overlapping non-specific bands (Fig. 5C) masking the lower SP3/DNA bands. These data support the results shown in Figure 5A and B which suggests that SP1-A, -B, -C, and -D oligonucleotides contain SP1- and SP3-binding sites. Taken together, these EMSAs demonstrate that SP1 and SP3 proteins are expressed in Caco-2 cells and bind to proximal GC-rich motifs in the *ELMO3* promoter.

SP1-BINDING SITES ARE IMPORTANT FOR ELMO3 PROMOTER ACTIVITY IN VITRO

The relevance of the four SP1-binding sites in the −270/−31 region for the promoter activity was addressed using transient transfection of *ELMO3* promoter/luciferase reporter gene constructs. Mutations were introduced in each SP1-binding site by site-directed mutagenesis with primers listed in Table I. Four basepair substitutions (A or T) were incorporated at the underlined conserved G/C core of these SP1-binding sites (GGGGCGGGG), as these substitutions have been shown to abolish SP1 binding as illustrated in (Fig. 5A,B) and in other studies [Shimakura et al., 2005, 2006]. Reporter constructs carrying mutated SP1-A (pGL4-ELMO3−1438/−31mutSP1-A), SP1-B (pGL4-ELMO3−1438/−31mutSP1-B), SP1-C (pGL4-ELMO3−1438/−31mutSP1-C), or SP1-D (pGL4-ELMO3−1438/

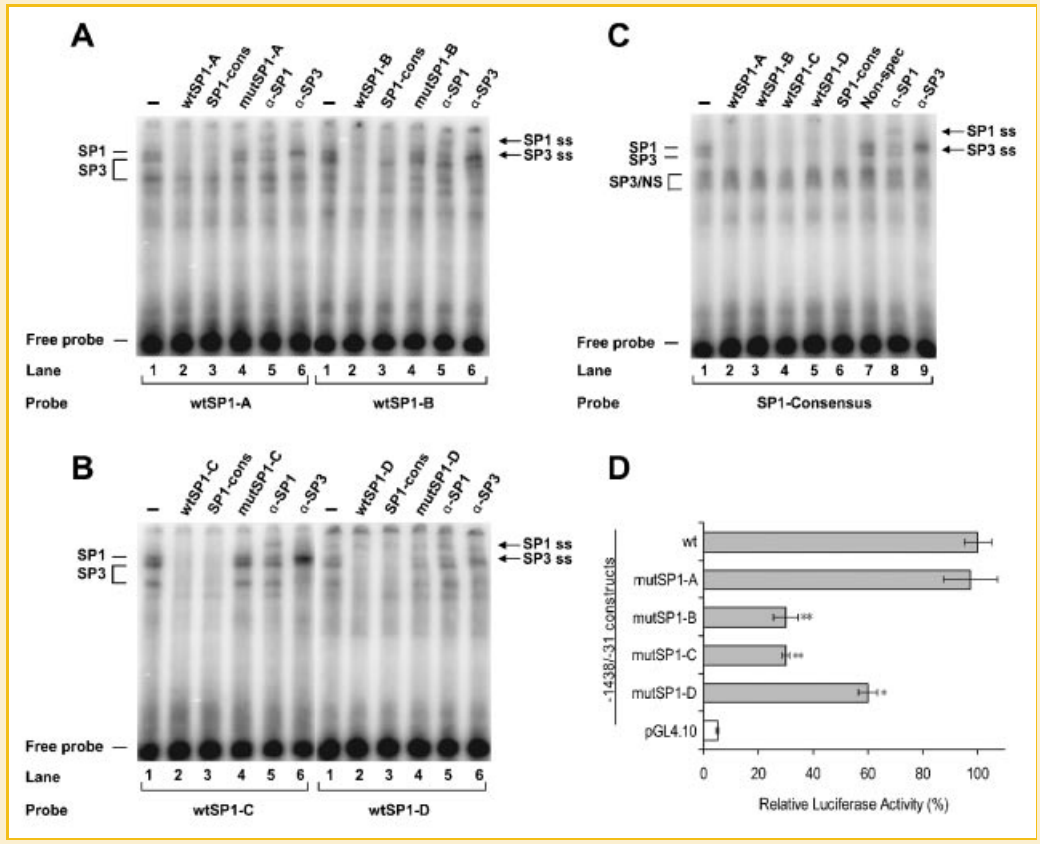

Fig. 5. EMSA with SP1 probes, and mutational analysis of SP1-binding sites. A: EMSA with double stranded wtSP1-A or wtSP1-B probes. Incubation of labeled wtSP1-A or wtSP1-B probes with Caco-2 nuclear extracts resulted in three shifted protein/DNA complex bands as indicated (lane 1). The competition assays were performed by adding a 100-fold excess of unlabeled wtSP1-A or wtSP1-B (lane 2), SP1-consensus (lane 3), and mutant oligonucleotides (lane 4). The addition of antibodies against SP1 (α-SP1) and SP3 (α-SP3) resulted in supershifted complexes indicated by arrows. B: EMSA with labeled wtSP1-C or wtSP1-D probes containing putative SP1 site C or D, respectively, was carried out with Caco-2 nuclear extracts. C: Incubation of a labeled consensus SP1 probes with Caco-2 nuclear extracts resulted in shifted protein/DNA complex bands (lane 1). The competition assays were performed by adding a 100-fold excess of unlabeled wtSP1-A (lane 2), wtSP1-B (lane 3), wtSP1-C (lane 4), wtSP1-D (lane 5), SP1-consensus (lane 6), and non-specific oligonucleotides (lane 7). The addition of α-SP1 and α-SP3 resulted in supershifted complexes indicated by arrows (lanes 8 and 9). NS, non-specific. D: Mutational analysis of SP1-binding sites in the CDX2 responsive region in the *ELMO3* promoter. The pGL4-ELMO3−1438/−31 constructs (200 ng) were transiently transfected into Caco-2 cells. Firefly luciferase activity was normalized to β-galactosidase activity. Data are reported as the relative luciferase activities in percentage compared to that of the wildtype and is present as the mean ± SD (N = 4). $^{*}P < 0.05$; $^{**}P < 0.001$, significantly different from the wildtype.

−31mutSP1-D) sites were generated and transfected into Caco-2 cells, and the relative luciferase activities were compared to that of the wildtype. As shown in Figure 5D, pGL4-ELMO3−1438/−31mutSP1-A did not affect the promoter activity. However, pGL4-ELMO3−1438/−31mutSP1-D significantly reduced the promoter activity by 40% ($P < 0.05$; Fig. 5D). Interestingly, mutation of SP1-B or SP1-C sites resulted in a significant decrease in the promoter activities of approximately 70% ($P < 0.001$). Taken together, these results point to an important transcriptional role of the SP1-binding sequences in the −270/−31 bp region. Especially, the SP1-B and -C sites seem to be important for the *ELMO3* promoter activity.

UP-REGULATION OF THE ELMO3 PROMOTER ACTIVITY BY CDX2 AND SP1 OVER-EXPRESSION

SP1 and CDX2 expression plasmids were co-transfected with pGL4−1438/−31 into Caco-2 cells to further investigate the regulatory role of these transcription factors for the *ELMO3* promoter activity. As shown in Figure 6, co-transfection analysis with pGL4-ELMO3−1438/−31, CDX2, or SP1 expressing plasmids in human Caco-2 cells showed that CDX2 alone caused a fourfold increase in the promoter activity and over-expression of SP1 alone caused a twofold increase in the activity. However, over-expression of SP1 showed a dose-dependent increase in the luciferase activity (data not shown). Thus, co-expression of CDX2 and SP1 resulted in a 10-fold increase of the *ELMO3* promoter activity (Fig. 6). Taken together the presented in vitro studies demonstrate that CDX2 and SP1 play important roles in regulating the *ELMO3* promoter activity. Based on the presented EMSAs (Fig. 5) and results of RNA interference studies in which SP1, SP3, and SP4 are knocked down individually [Higgins et al., 2006; Abdelrahim et al., 2007], it is likely that SP3 and SP4 contribute to the expression of ELMO3.

CDX2- and SP1-mediated regulation of gene expression in the intestine has been reported previously [Shimakura et al., 2005]. The expression of the H^{+}-coupled peptide transporter 1 (*PEPT1*) has been shown to be regulated by CDX2 and SP1 and that a synergism between CDX2 and SP1 in activation of *PEPT1* promoter

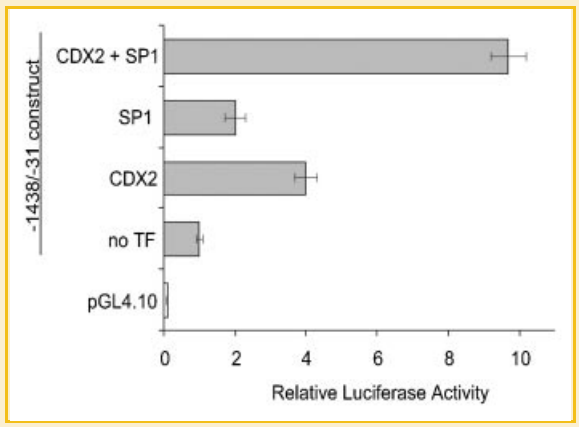

Fig. 6. Activation of the *ELMO3* promoter by CDX2 and SP1. Caco-2 cells were transiently transfected with 100 ng of the pGL4-ELMO3−1438/−31 construct and the expression vector for CDX2 (100 ng), and/or SP1 (800 ng). The total amount of transfected DNA was kept constant by adding empty vector. Data are reported as the relative fold-activation relative to the pGL4-ELMO3−1438/−31 construct transfected alone and represent the mean ± SD (N = 4).

using co-expression experiments exists [Shimakura et al., 2005, 2006]. It was furthermore shown that CDX2 and SP1 are bound to *PEPT1* promoter in vivo in Caco-2 cells by ChIP [Shimakura et al., 2006]. Thus, the combination of CDX2- and SP1-binding elements seems to direct specific expression in intestinal epithelial cells.

The intestine-specific transcription factor CDX2 has been implicated as a critical regulator for the gene expression during mammalian development, differentiation, and cancer [Duprey et al., 1988; Freund et al., 1998; Gao et al., 2009]. It plays multiple roles in both normal development and the suppression of cancer [Bonhomme et al., 2003]. However, other studies have raised the question of whether CDX2 has a positive or negative effect in wound healing and cell migration [Rao et al., 1999; Gross et al., 2008]. On the other hand, SP1, a zinc finger type of transcription factor, plays important roles in a variety of pathophysiological processes including cell cycle regulation, growth regulation, cell survival, and metastasis of cancer [Grinstein et al., 2002; Abdelrahim et al., 2004]. Little is known about the migration processes along crypt–villus axis in intestines. Loss of accurate control of cell migration and cell shedding presumably cause serious disruption of the mucosal architecture. Based on the data presented here that CDX2 regulate the activity of the *ELMO3* promoter in Caco-2 cells, we hypothesize that *ELMO3* contributes with important functions in the highly controlled migration process in the crypt–villus. In summary, it may be possible that CDX2 physically and functionally interacts with SP1 and associates with the *ELMO3* promoter to play an important role in the renewing processes within the intestinal epithelia and cancer.

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