Different effects of the *Cdx1* and *Cdx2* homeobox genes in a murine model of intestinal inflammation

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Aims: The CDX1 and CDX2 homeoproteins are intestine-specific transcription factors regulating homeostasis. We investigated their relevance in experimentally-induced intestinal inflammation.

Methods: The response to intestinal inflammation induced by dextran sodium sulfate (DSS) was compared in wild type, *Cdx1*^{-/-} and *Cdx2*^{+/-} mice. Intestinal permeability was determined in wild type and *Cdx2*^{+/-} mice. Protein-protein interactions were investigated by co-immunoprecipitation and GST-pulldown, and their functional consequences were assessed using Luciferase reporter systems.

Results: Heterozygous *Cdx2*^{+/-} mice, but not *Cdx1*^{-/-} mice, were hypersensitive to DSS-induced acute inflammation as all these mice showed blood in the stools at day 1 of DSS treatment. Hypersensitivity was associated to a 50% higher intestinal permeability. In *Cdx2*^{+/-} mice, the colonic epithelium was repaired during the week after the end of DSS treatment, whereas two weeks were required for wild type animals. Subsequently, no colonic tumour was observed in *Cdx2*^{+/-} mice subjected to 5 repeated cycles of DSS, in contrast to the 2.7 tumours found per wild type mouse. Based on the fact that *Smad3*^{+/-} mice, like *Cdx2*^{+/-} mice, better repair the damaged intestinal epithelium, we found that the CDX2 protein interacts with SMAD3, independently of SMAD4, resulting in a 5-fold stimulation of SMAD3 transcriptional activity. CDX1 also interacted with SMAD3 but it inhibited by 10-fold the SMAD3/SMAD4-dependent transcription.

Conclusion: The *Cdx1* and *Cdx2* homeobox genes have distinct effects on the outcome of a pro-inflammatory challenge. This is mirrored by different functional interactions of the CDX1 and CDX2 proteins with SMAD3, a major element of the TGFβ signalling pathway.

Chronic bowel inflammation gives rise to high morbidity diseases characterised by successive periods of exacerbation and remission.¹ It also increases the risk of cancer in relation with disease duration and early onset.^{2–3} Inflammatory bowel diseases depend on altered interactions between immunoregulatory factors in the gut mucosa and environmental factors.⁴ The intestinal epithelium, which is disrupted and repaired during ulceration and remission, participates in these interactions at the interface between the mucosa and the luminal content.

CDX1 and CDX2 are homeodomain transcription factors specific of the intestinal epithelium. During gut development, the *Cdx2* gene dictates intestinal identity⁵ whereas *Cdx1* has a less prominent role.⁶ *Cdx1* and *Cdx2* have both similar and distinct effects on proliferation and differentiation in intestinal cell lines,^{7–9} and also in pathological conditions. For instance, the reduction of *Cdx2* expression in *Cdx2*^{+/-} mice facilitates the progression of colorectal cancer induced chemically or linked to a genetic predisposition, suggesting a tumour suppressor role.^{10–11} In contrast, the loss of *Cdx1* reduces the hyperproliferative state induced by pRb/p300 deficiency.¹²

Since the intestinal epithelium is perturbed and repaired in inflammatory bowel diseases, we used *Cdx1*^{-/-} and *Cdx2*^{+/-} mice and the experimental model of colitis induced by dextran sodium sulfate (DSS) to investigate the role of the *Cdx1* and *Cdx2* genes on the outcome of the intestinal epithelium after pro-inflammatory injury.

MATERIAL & METHODS

Mice treatments and cell cultures

Cdx2^{+/-},¹³ *Cdx1*^{-/-}⁶ and wild type mice in the 129sv/C57Bl6 background were housed under pathogen-free conditions and given treatments according to the guidelines of the Ethic

Committee of the University Louis Pasteur of Strasbourg. For acute treatments, two month-old animals received 3% DSS (36–50 kDa, MP Biomedicals, Illkirch, France) in drinking water for five days and then tap water ad libitum. For chronic treatments, mice were subjected either to three cycles consisting of five days with 1.5% DSS in drinking water followed by 16 days with tap water, or to five cycles of five days with 3% DSS followed by 16 days with tap water. Blood in the stools was analysed using Haemocult II® (SKD, Gagny, France). Mice were euthanised by cervical disruption. The gastro-intestine was removed, flushed with PBS, mounted as Swiss Roll, fixed in 4% paraformaldehyde for 4 h and embedded in paraffin. Assessment of inflammation of the colon was performed in a blinded fashion by two pathologists with regard to stiffness, edema, ulcerations and thickness, as described.¹⁴

T84, HCT116 (ATCC) and HCT116 *Smad4*^{-/-} human colon cancer cells¹⁵ were cultured under standard conditions in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C in humidified atmosphere under 5% CO₂.

In vivo intestinal permeability

Intestinal permeability was determined in three-month old wild type (n = 12) and *Cdx2*^{+/-} (n = 12) mice by measuring the appearance of FITC-dextran in the blood.¹⁶ Mice starved for 24 h received either tap water or 3% DSS in drinking water (6 mice per genotype) for 16 h. Then, FITC-dextran (4.4 kDa; Sigma, St Quentin Fallavier, France) was administered by gavages (60 mg/100 g body weight). Blood was collected 4 h later by orbital retrobulbar puncture and centrifuged at 3000 g for 20 min at 4°C. Plasma (50 µL) was diluted volume to

Abbreviation: DSS, dextran sodium sulfate

volume with PBS to determine fluorescence using a Molecular FX Imager (Biorad, Marnes-la-Coquette, France).

Immunohistochemistry

Immunohistochemistry was performed on 5 µm paraffin sections¹¹ using primary antibodies raised against Ki67 (dilution 1:50, ab833, Abcam, Cambridge, UK), β-catenin (dilution 1:150, clone 14, BD Transduction Laboratories, Lexington, Kentucky, USA), Cdx2 (dilution 1:500, CDX2-88, Biogenex, San Ramon, California, USA) and Cdx1.¹¹

Plasmids

The plasmids pCdx2-S, pCdx1-S, pCB6-HA-CDX2 and pCB6-HA-CDX1,^{7,17} those encoding 6xMyc-Smad1, -2, -3, -4, -6 in pCDNA3.1 and Flag-Smad3-MH1-L-MH2, -MH1, -MH1-L, -L-MH2, -MH2 in pDEF3¹⁸ and the reporter vectors CAGA-Luc¹⁹ and pSI-Luc²⁰ have been described. To construct pGST-CDX2 and pGST-CDX1, a NheI site was introduced immediately downstream of the translation start site of pCdx1-S and pCdx2-S,¹⁷ the resulting plasmids were cut with NheI, filled in with T4 DNA polymerase, then cut with BamHI, and the fragments were introduced into the EcoRV/BamHI sites of pBC.²¹

Co-immunoprecipitation, GST-pulldown

T84 or HCT116 cells cultured at 70–80% confluence were transfected using Jet-PEI (Polyplus Transfection, Illkirch, France) for 48 h, then scraped in ice-cold NP40 lysis buffer (20 mM Tris at pH7.5, 150 mM NaCl, 1% Igepal) and a cocktail of protease inhibitors (Sigma), placed on ice for 10 min and centrifuged at 10,000 g for 10 min at 4°C. Protein content in the supernatant was assayed using Bradford reaction. For co-immunoprecipitation or GST-pulldown assays, protein extracts (1 mg) were incubated overnight at 4°C with agarose bead-coupled anti-HA antibody matrix (25 µl, 3F10, Roche, Indianapolis, IN) or for 3 h under gentle agitation at 4°C with glutathione-Sepharose-4B beads (25 µl, 50% vol/vol, GE Healthcare, Munich, Germany), then beads were washed three times for 15 min in lysis buffer and proteins were analysed by SDS-PAGE and western blot. Western blots were performed as described²² using primary antibodies against HA (3F10, Roche, dilution 1:1000), Flag (M2, Sigma, dilution 1:1000), Myc (9E10, Santa Cruz, Heidelberg, Germany, dilution 1:1000), GST (G-7781, Sigma, dilution 1:5000), CDX2 (CDX2-88, Biogenex, dilution 1:5000) or SMAD3 (ab28379-100, Abcam, dilution 1:1000), and secondary antibodies coupled to horseradish peroxidase (GE Healthcare): sheep anti-mouse κ-chain immunoglobulins (dilution 1:5000), donkey anti-rabbit immunoglobulins (dilution 1:5000) or sheep anti-rat immunoglobulins (dilution 1:5000). Co-immunoprecipitation and GST-pulldown assays were repeated at least three times.

Luciferase assays

Luciferase assays were performed in HCT116 or HCT116 Smad4^{-/-} cells co-transfected with the indicated luciferase reporter plasmids and with pRL-null (Promega, Charbonnières, France) to normalise transfection efficiency.²² Firefly luciferase activity was normalised with renilla luciferase activity and the data were expressed as fold of induction in the experimental condition as compared to the reporter control. Luciferase assays were repeated at least three times in triplicate.

Statistical analyses

Data were reported as mean (SEM) and analysed with the unpaired Student t test. P values <0.05 were considered statistically significant.

RESULTS

Cdx2^{+/-} but not Cdx1^{-/-} mice are hypersensitive to DSS induced inflammation

Cdx1^{-/-}, Cdx2^{+/-} and wild type mice were given 3% DSS in drinking water for five days and then allowed to recover with tap water. As generally observed with this experimental model, mortality by sepsis occurred in 10–15% of the animals. As shown in fig 1A, no blood appeared in the stools of wild type and Cdx1^{-/-} mice before day two of treatment. At day two, blood was observed in less than 25% of wild type and Cdx1^{-/-} mice and the percentage progressively rose up to day five. In contrast, all Cdx2^{+/-} heterozygotes exhibited blood in the stools as early as day one of treatment. At day one, structural alterations including edema and crypt atrophy already occurred in the colon of Cdx2^{+/-} mice (fig 2Aa,b), in contrast to wild type and Cdx1^{-/-} mice (fig 2Ac). At day five, the colonic mucosa was severely impaired with gut wall thickening, massive round cell infiltration, edema and areas of epithelial denudation, irrespective of the genotype (not shown).

The five days treatment with DSS induced a progressive body weight loss. Consistent with the precocious appearance of blood in the stools in Cdx2^{+/-} mice, body weight loss peaked two days after the end of DSS treatment in these animals (day seven) but the peak was delayed by four days in wild type and Cdx1^{-/-} mice (day 11) (fig 1B). Consequently, body weight recovery was faster in Cdx2^{+/-} than in wild type and Cdx1^{-/-} mice (fig 1B). In

Figure 1 Blood in the stools and body weight loss after acute dextran sodium sulfate (DSS) treatment. (A) Percentage of animals positive for the presence of blood in the stools using the Haemoccult test during acute DSS treatment for five days. Open, grey and black boxes correspond respectively to wild type, Cdx1^{-/-} and Cdx2^{+/-} mice (n>10 per genotype at each time point). (B) Percentage of body weight loss during the 5-day treatment with DSS (grey box) and during the following 20 days of recovery. Average data are given for n>5 per genotype at each time point: wild type, Cdx1^{-/-} and Cdx2^{+/-} mice. In A and B, SEM has been omitted for clarity. * indicates p<0.01 between Cdx2^{+/-} mice on one hand and wild type and Cdx1^{-/-} mice on the other hand.

Figure 2 Histology of the distal colon during acute dextran sodium sulfate (DSS) treatment and recovery. (A) Alteration of the colon mucosa after acute DSS treatment in $Cdx2^{+/-}$ mice. $Cdx2^{+/-}$ mice treated for 1 day exhibit edema (asterisk) (a) and crypt atrophy (b), whereas the mucosa remains unaltered in wild type and $Cdx1^{-/-}$ animals (c). Bar is 100 μ m. (B) Recovery after acute DSS treatment in $Cdx2^{+/-}$ and wild type mice. Histology of the distal colon mucosa of wild type (a-c) and $Cdx2^{+/-}$ mice (d-f), 1 day (a,d), 3 days (b,e) and 8 days (c,f) after the end of DSS administration. The inset in d is a higher magnification showing the granulation tissue. Bar is 100 μ m in a,b,d,e and 200 μ m in c,f.

wild type animals, severe mucosal alterations were observed at days six and eight (fig 2Ba,b) and regenerative epithelium appeared only in a third of the colonic surface at day 13 (fig 2Bc). In contrast, regenerative epithelium was already apparent at day six and day eight in $Cdx2^{+/-}$ heterozygotes (fig 2Bd,e) and the major part of the colonic surface was repopulated by structurally normal epithelium by day 13 (fig 2Bf). Differences between $Cdx2^{+/-}$ and wild type mice were confirmed by the inflammatory score (fig 3).

Reduced *Cdx2* expression increases intestinal permeability

The hypersensitivity of $Cdx2^{+/-}$ mice to acute treatment with DSS, which is an abrasive agent, prompted us to compare intestinal permeability in wild type and $Cdx2^{+/-}$ mice by measuring the appearance of FITC-dextran in blood after gavages. By this way, FITC-dextran is absorbed at the level of

the small intestine and colon.²³ In basal condition, intestinal permeability was higher in $Cdx2^{+/-}$ than in wild type mice (fig 4A). Permeability was also higher after a short exposure to 3% DSS for 16 h (fig 4B). Unlike $Cdx2^{+/-}$ mice, intestinal permeability was twofold lower in $Cdx1^{-/-}$ compared to wild type animals (not shown).

Sensitivity to colorectal cancer linked to chronic inflammation by DSS depends on *Cdx2*

Despite their hypersensitivity to the inflammatory stress resulting from acute DSS treatment, $Cdx2^{+/-}$ mice exhibit a better recovery than wild type and $Cdx1^{-/-}$ mice at the end of the pro-inflammatory challenge. Therefore, we compared the behaviour of $Cdx2^{+/-}$ and wild type mice after chronic inflammation induced by repeated administration of DSS, to mimic sequences of exacerbation and remission. In a pilot experiment, 10 wild type and 10 $Cdx2^{+/-}$ mice were subjected

Figure 3 Inflammatory scores. Inflammatory scores were determined in wild type (open boxes) and $Cdx2^{+/-}$ mice (grey boxes). *indicates $p < 0.01$ for $n = 5$ animals.

Figure 4 Intestinal permeability. Wild type (light grey boxes) and $Cdx2^{+/-}$ mice (dark grey boxes) were given water at libitum (a) or 3% DSS for 16 h (b) and then FITC-dextran by gavages. The amount of FITC-dextran in blood was measured 4 h later. Data are given SEM for $n = 6$ animals.

to three cycles of DSS administration, each comprising five days with 1.5% DSS in drinking water followed by a 16-day recovery period. Due to the mortality by sepsis at each cycle, five mice of each genotype survived the DSS treatments and were killed 10 weeks later for histological examination. Wild type mice, but not $Cdx2^{+/-}$ animals, exhibited edema in the colon, and there was an adenomatous polyp in the distal colon of one wild type mouse (not shown). Then, we repeated the cyclic treatment more drastically using 15 wild types and 15 $Cdx2^{+/-}$ mice subjected to five cycles of DSS at 3%. Three wild types and 4 $Cdx2^{+/-}$ mice survived and were sacrificed 12 weeks after the end of the DSS treatment. $Cdx2^{+/-}$ heterozygotes exhibited regular crypt architecture with a low rate of immune cells infiltration throughout the colon (fig 5Aa). In contrast, the colonic mucosa of wild type mice showed substantial infiltration of immune cells together with multiple areas characterised by a disorganised mucosal architecture. These areas appeared as flat epithelium above granular tissue, dilated mucinous glands and clusters of irregular crypts (fig 5Ab-d). Thus, unlike $Cdx2^{+/-}$ mice, wild type mice showed imperfect repair of the colonic epithelium upon chronic DSS-induced colitis.

Next, we investigated the long-term consequences of chronic inflammation using a new series of 40 mice, 20 of each

genotype, subjected to five cycles of treatment with 3% DSS and sacrificed 50 weeks later. Six wild types and five $Cdx2^{+/-}$ animals survived the DSS treatment. Macroscopic examination at necropsy failed to detect any tumour in the small intestine and colon of $Cdx2^{+/-}$ animals. By contrast, five out of the six wild type mice showed tumours in the colon, giving a total of 16 tumours (fig 5Ba). Tumours corresponded to adenomatous polyps with low- to high-grade dysplasia or adenocarcinoma that invaded the lamina propria into the submucosa (fig 5Bb). Tumour cells were highly proliferative as shown by strong Ki67 immunolabelling (fig 5Bc). The expression of several proteins was altered: β -catenin distribution was heterogeneous with some cells exhibiting a predominant membranous localisation and others showing cytoplasmic/nuclear β -catenin accumulation (fig 5Bd); CDX2 and CDX1 proteins expression was reduced and heterogeneous in the tumours compared to normal adjacent mucosa (fig 5Be,f).

Functional interaction between the proteins CDX2 and SMAD3

Similarly to $Cdx2^{+/-}$ mice which better repair the damaged colon epithelium after DSS injury, accelerated re-epithelialisation of the colon characterises $Smad3^{+/-}$ mice in a model of

Figure 5 Chronic dextran sodium sulfate (DSS) treatment in $Cdx2^{+/-}$ and wild type mice. (A) Mice treated for five cycles of DSS and sacrificed 12 weeks later. Histological examination of the colon mucosa revealed regular crypt architecture in $Cdx2^{+/-}$ heterozygotes (a), but disorganised area in wild type animals (b-d). Bar is 100 μ m. (B) Mice treated for five cycles of DSS and sacrificed 50 weeks later. (a) Examples of tumours developing in the colon of wild type mice. (b) Histology of a colon tumour. Tumour sections were immunostained with antibodies against Ki67 (c), β -catenin (d), CDX2 (e) and CDX1 (f). In (d) the arrowhead shows tumour cells with membranous β -catenin and the arrow indicates tumour cells with cytoplasmic/nuclear β -catenin. The inset in (e) and the arrowhead in (f) respectively show CDX2 and CDX1 immunostaining in the normal adjacent colon epithelium. The sparse staining in the lamina propria in (e) corresponds to the binding of secondary anti-mouse antibody to immune cells. Bar is 5 mm in (a), 500 μ m in (b) and 100 μ m in (c-f).

colitis.²⁴ Therefore, we analysed the possibility for a connection between Cdx2 and Smad's in intestinal cells. For this purpose, HCT116 cells were transfected with the reporter plasmid CAGA-Luc to monitor SMAD transcriptional activity (fig 6Aa). Co-transfection of CAGA-Luc with plasmids encoding either SMAD3 (without co-transfection with SMAD4) or CDX2 led respectively to a modest two-fold and 3.5-fold increase of luciferase activity. However, luciferase activity was 10-fold stimulated when both plasmids for SMAD3 and CDX2 were combined, and the effect was proportional to the amount of CDX2 (fig 6Ab). The synergistic effect of SMAD3 and CDX2 on CAGA-Luc was independent of the co-stimulator of SMAD3, SMAD4, since it was also effective in HCT116 Smad4^{-/-} cells which lack SMAD4 expression (fig 6Ab). Next, instead of the CAGA-Luc reporter, the experiments were performed using the CDX2-responding reporter plasmids SI-Luc (fig 6Ac) and LPH-Luc (not shown) containing the promoters of the intestinal genes for sucrase-isomaltase and lactase-phlorizin hydrolase. The transcriptional activity of both promoters was stimulated by CDX2, whereas SMAD3 alone or in combination with SMAD4 was without effect. Interestingly, the combination of CDX2 and SMAD3 did not further activate transcription over CDX2 alone. Therefore, SMAD3 and CDX2 functionally cooperate to stimulate the transcription of a SMAD-responsive reporter, but not of CDX2-responsive reporters.

In an attempt to address the molecular basis of the collaborative effect between CDX2 and SMAD3, T84 cells, which have an unaltered TGF β signalling pathway, were transfected with the plasmid expressing HA-tagged CDX2. Co-immunoprecipitation experiments with anti-HA antibody led to recover endogenous SMAD3 with HA-tagged CDX2 (fig 6Ba). To confirm this result, HCT116 cells were co-transfected with the plasmids encoding the GST-CDX2 fusion protein and Myc-SMAD3. GST-CDX2 was able to pulldown Myc-SMAD3 (fig 6Bb), indicating that CDX2 and SMAD3 belong to a same protein complex. Myc-SMAD2 and Myc-SMAD4 were also pulled-down by GST-CDX2, but not Myc-SMAD1, suggesting that CDX2 differentiates between members of the SMAD family (data not shown). The SMAD3 protein is made of the MH1 domain responsible for nuclear import, DNA binding and transcription, a linker domain and the MH2 domain responsible for oligomerisation and transcription. By pull-down assays in HCT116 cells with plasmids encoding GST-CDX2 and Flag-tagged versions of truncated forms of SMAD3, we identified MH2 as the domain of interaction with CDX2 (fig 6Bc). Reciprocally, we constructed plasmids encoding either the amino-terminal transactivating domain of CDX2 or the DNA-binding homeodomain linked to the carboxy-terminal domain, but none of these truncated forms co-purified with SMAD3, in contrast to the full-length CDX2, suggesting that a complex conformation of CDX2 is required for interacting with SMAD3 (data not shown).

Different effects of CDX1 and CDX2 proteins on SMAD3

Having demonstrated the interaction between SMAD3 and CDX2, we tested the homologous homeodomain factor CDX1. Cotransfection of HCT116 cells with plasmids encoding GST-CDX1 and Myc-SMAD3 revealed an interaction between SMAD3 and CDX1 by GST-pulldown (fig 8Aa). Like SMAD3, SMAD4 also interacted with GST-CDX1 (not shown). Using Flag-tagged truncated forms of SMAD3 we identified the domain MH2 of SMAD3 for interaction with CDX1, as previously shown for CDX2 (fig 7Ab).

Then we examined the functional effect of the SMAD3/CDX1 interaction. Upon co-transfection in HCT116 cells, CDX1 did not change the low transcriptional activity of SMAD3 on the CAGA-Luc reporter plasmid (not shown). Since SMAD3 cooperates with SMAD4 for optimal transcriptional activation,

we analysed the effect of CDX1 on the SMAD3/SMAD4 heterodimer. Co-transfection of CAGA-Luc with the plasmids encoding SMAD3, SMAD4 and CDX1 showed that CDX1 inhibited SMAD3/SMAD4-dependent transcription of CAGA-Luc (fig 7Ba) in a dose-dependent manner (fig 7Bb). The inhibitory effect of CDX1 was comparable to that exerted by SMAD6 (fig 7Ba).

Since CDX1 interacts with SMAD3/SMAD4 to inhibit the activity of this complex whereas CDX2 interacts with SMAD3 to stimulate its function, we asked if CDX2 can release the inhibition exerted by CDX1 on SMAD3/SMAD4. As illustrated in fig 7Ca, the addition of CDX2 counteracted the inhibitory effect of CDX1 on SMAD3/SMAD4. The opposing effect exerted by CDX2 on the inhibition by CDX1 was not caused by an interaction between CDX1 and CDX2 since GST-CDX2 did not pulldown HA-CDX1 and reciprocally, GST-CDX1 did not pulldown HA-CDX2 (fig 7Cb).

DISCUSSION

This study demonstrates that the intestine-specific homeobox genes *Cdx1* and *Cdx2* have different impacts on the response of the intestinal epithelium to a pro-inflammatory stress, since *Cdx1*^{-/-} mice behave like wild type animals, whereas *Cdx2*^{+/-} mice exhibit both hypersensitivity to DSS-induced colitis and improved mucosal repair.

The transcription factor CDX2 is at the head of the intestinal genetic program⁵ and controls the expression of a number of downstream genes, some of which being thought to play a role in inflammation. For instance, CDX2 is a positive regulator of the *Muc2* and *TFF3* genes,^{25, 26} whose deficiency induces hypersensitivity to DSS-induced colitis.^{26, 27} Moreover, CDX2 controls cell-cell interactions and the expression of cadherins^{7, 28, 29} In line with this, evidence is provided here that *Cdx2*^{+/-} mice exhibit increased intestinal permeability, which itself could strengthen the abrasive effect of DSS and exacerbate the acute inflammatory response. Therefore, it is likely that a reduced expression of *Cdx2* changes several functions and properties of the colon epithelium, which together converge towards the phenotype of hypersensitivity of *Cdx2*^{+/-} mice to DSS-induced colitis.

Epithelial repair after injury requires a complex interplay between cell migration and proliferation to resurface the mucosa and to restore crypt morphology. *Cdx2* regulates the proliferation/differentiation balance.^{7, 9} Moreover, we found that decreasing *Cdx2* expression enhances the migration of intestinal cells,³⁰ which may account for the improved intestinal repair observed in *Cdx2*^{+/-} mice. Like *Cdx2*^{+/-} mice, *Smad3*^{+/-} animals also show a better intestinal recovery after experimental colitis,²⁴ while the inhibition of regulatory SMADs appears as a general condition to accelerate healing in several epithelia.^{31, 32} The improved recovery of the colonic epithelium after inflammatory injury in *Cdx2*^{+/-} mice, as in *Smad3*^{+/-} animals, is of significance since we report a protein/protein interaction between CDX2 and SMAD3 to synergistically stimulate SMAD3 transcriptional activity. Hence, the better healing of *Cdx2*^{+/-} mice may result from the fact that reducing CDX2 subsequently diminishes the activity of SMAD3, therefore improving tissue repair. SMAD3 associates with SMAD4 to transduce TGF β signals, but TGF β receptors also signal via SMAD-independent pathways. Of note, blocking TGF β signalling by a dominant-negative receptor slows down intestinal repair after inflammatory injury,³³ in clear opposition with the phenotype of *Smad3*^{+/-} mice.²⁴ This suggests that signalling cascades downstream of TGF β receptors must be correctly balanced between SMAD-dependent and SMAD-independent pathways during tissue repair. In this context, CDX2 may regulate this balance by binding and regulating the activity of SMAD3. In addition, we show here that CDX1, the homologue of CDX2, also binds SMAD3, yet inhibiting the SMAD3/SMAD4

Figure 6 Functional interaction between the CDX2 and SMAD3. (A) Transcriptional effect of CDX2 on SMAD3. In these transfection experiments, CAGA-Luc and SI-Luc report downstream responses to SMAD3 and to CDX2, respectively. (a) HCT116 cells were transfected with the reporter plasmid CAGA-Luc and with the plasmids encoding CDX2 or SMAD3 or CDX2 + SMAD3 or the control vector pCB6. (b) HCT116 (black boxes) or HCT116-Smad4^{-/-} cells (grey boxes) were co-transfected with the reporter plasmid CAGA-Luc together with a constant amount of SMAD3-encoding plasmid (0.5 µg) and an increasing amount of CDX2-encoding plasmid (0–1.2 µg). (c) HCT116 cells were transfected with the reporter plasmid SI-Luc and plasmids encoding CDX2, SMAD3 and SMAD4. Results are expressed as fold of induction compared to cells transfected with the control plasmid pCB6. (B) Molecular interaction between CDX2 and SMAD3. (a) T84 cells were transfected with the plasmid encoding HA-CDX2 or with the control plasmid pCB6. Proteins immunoprecipitated with anti-HA antibody were controlled by western blots using anti-HA antibody and assayed for SMAD3 with anti-SMAD3. The presence of SMAD3 in cell extracts prior to HA-immunoprecipitation was controlled using anti-SMAD3 antibody (Input). (b) HCT116 cells were co-transfected with the plasmids encoding Myc-SMAD3 and GST-CDX2 or the control vector, pBC. GST-pull-down extracts were analysed by western blots using anti-GST antibody and assayed for the presence of Myc-SMAD3 using anti-Myc. Western blot with anti-Myc confirmed the presence of Myc-SMAD3 in cell extracts prior to GST-pull-down (Input). (c) HCT116 cells were transfected with the plasmids encoding GST-CDX2 and Flag-tagged truncated versions of SMAD3 and analysed after GST-pull-down as in (b) using anti-GST and anti-Flag antibodies.

complex, in contrast to the stimulatory effect exerted by CDX2 on SMAD3. The opposing effects of CDX1 and CDX2 are in line with the different response of *Cdx1*^{-/-} and *Cdx2*^{+/-} mice to DSS-induced colitis. CDX2 and CDX1 have overlapping and different effects on cell proliferation and differentiation,^{7–9} and we have previously reported that these two factors can differentially interact with components of the basic transcriptional machinery.³⁴ Here, the binding of CDX2 and CDX1 to a common partner, SMAD3, while giving opposite effects, provides a new molecular mechanism to explain the divergent functions of these two homologous factors.

A tumour suppressor role has been attributed to *Cdx2* since *Cdx2*^{+/-} mice are hypersensitive to colon carcinogenesis induced chemically by AOM or linked to a genetic predisposition.^{10–11} Paradoxically, we found here that *Cdx2*^{+/-} mice are resistant to colon cancers induced by chronic inflammation. AOM is a mutagenic compound, whereas DSS is a non-mutagenic abrasive agent that compromises cell proliferation.³⁵ Hence, CDX2 reduction has ultimately opposite consequences on colorectal cancer, depending on the mechanism of tumourigenesis. It is likely that the improved tissue repair in *Cdx2*^{+/-} mice protects them from prolonged inflammation and subsequent tumour development. Reducing CDX2 could also attenuate prolonged inflammatory stress as it is a regulator of the peptide transporter PEPT1/SLC15A1³⁶ involved in the intake of bacterial peptides responsible for the production of accessory immune molecules.³⁷ The sequence from chronic inflammation

to cancerous evolution remains to be thoroughly explored. Chronic inflammation produces genotoxic reactive oxygen and nitrogen species for epithelial cells.³⁸ It may also induce the recruitment of bone marrow-derived stem cells that contribute to tumourigenesis, as recently shown in a murine model of gastric cancers.³⁹

In conclusion, we report here a crucial role for *Cdx2* in inflammation-induced disruption and repair of the colonic epithelium and subsequently in colon cancer induced by chronic inflammation. *Cdx1*^{-/-} mice, unlike *Cdx2*^{+/-} animals, are not hypersensitive to acute inflammation, revealing different functions of these two homeobox genes in the gut. The present study also uncovers a connection between the CDX1 and CDX2 proteins and members of the SMAD family of mediator of the BMP/TGFβ pathways. In line with this, the different effects of the *Cdx1* and *Cdx2* homeobox genes in the model of intestinal inflammation by DSS are mirrored by different effects of the CDX2 and CDX1 proteins in interaction with SMAD3.

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Figure 7 Functional interaction between CDX1 and SMAD3/SMAD4. (A) Molecular interaction of CDX1 and SMAD3. (a) HCT116 cells were co-transfected with the plasmids encoding Myc-SMAD3 and either GST-CDX1 or the control plasmid pCB. (b) HCT116 cells were transfected with plasmids encoding GST-CDX1 and Flag-tagged truncated versions of SMAD3. After GST-pull-down, western blot analyses were performed as described in fig 6B using anti-GST, anti-Myc or anti-Flag antibodies. (B) Functional interactions between CDX1 and SMAD3/SMAD4. (a) HCT116 cells were transfected with the vector CAGA-Luc and with the plasmids for SMAD3, SMAD4, SMAD6, CDX1 and/or the control plasmid pCB6, as indicated. (b) HCT116 cells were co-transfected with CAGA-Luc together with a constant amount of SMAD3- + SMAD4-encoding plasmids (0.5 μ g) and an increasing amount of CDX1-encoding plasmid (0–0.5 μ g). Results are expressed as fold of induction compared to cells transfected with the control plasmid pCB6. (C) Different effects of CDX1 and CDX2. (a) HCT116 cells were transfected with the CAGA-Luc plasmid and the indicated plasmids encoding CDX1, CDX2, SMAD3 and/or SMAD4. Results are expressed as fold of induction compared to cells transfected with the control plasmid pCB6. (b) Cells were transfected either with the plasmids encoding GST-CDX1 and HA-CDX2 or with the plasmids encoding GST-CDX2 and HA-CDX1. GST-pull-down extracts were analysed by western blots using anti-GST antibody and assayed for the presence of HA-tagged proteins using anti-HA antibody. The presence of respectively HA-CDX1 or HA-CDX2 in the cell extracts prior to GST-pull-down was controlled by western blot using anti-HA antibody (Input).

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From questions on page 1684

This patient has sarcoidosis with involvement of the stomach.

Gastroscopy showed an irregularity at the Z-line with an odd appearance on retroversion and biopsies were indicative of granulomatous gastritis. Blood tests, including serum angiotensin converting enzyme, were normal. Widespread lymphadenopathy as well as pulmonary nodules were noted at CT scan and a cervical lymph node biopsy showed non-caseating granulomata consistent with a diagnosis of sarcoidosis. His chest symptoms and lymphadenopathy responded to treatment with prednisolone and gastrointestinal haemorrhage has not recurred.

The differential diagnosis of granulomatous gastritis includes sarcoidosis, tuberculosis, Crohn's disease, histoplasmosis, syphilis, underlying malignancy, vasculitis or a foreign body reaction. Symptomatic gastrointestinal sarcoidosis is uncommon and usually occurs in the context of established sarcoid or as a result of disseminated disease. The commonest site is gastric, though involvement of the oesophagus, small bowel and colon have also been reported. Gastric sarcoid may present with epigastric pain, vomiting, weight loss and haemorrhage, and endoscopic appearances include localised or diffuse hyperaemia, ulceration, atrophic gastritis and mucosal thickening. Corticosteroids are the first-line treatment for symptomatic sarcoidosis.

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