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Isabelle Hinkel

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ÉCOLE DOCTORALE Des sciences de la vie et de la Santé

Inserm U682, De l'homéostasie tissulaire au cancer et à l'inflammation

THÈSE présentée par :

Isabelle HINKEL

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Discipline/ Spécialité : Aspects Moléculaires et Cellulaires de la Biologie

**Plasticité des Cellules Cancéreuses Coliques:
Impact du Facteur d'Identité Tissulaire Cdx2**

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Résumé de thèse en français

GENERAL INTRODUCTION

I) The intestinal epithelium

1. Structure and function of the intestine

The intestine is composed of two main functional segments: the small intestine (subdivided into duodenum, jejunum and ileon) and the colon. The primary function of the small intestine is the absorption of nutriment, whereas in the colon water and salts are extracted and the stool is compacted. Histologically, the intestine is composed of different concentric layers as detailed on **Figure 1**.

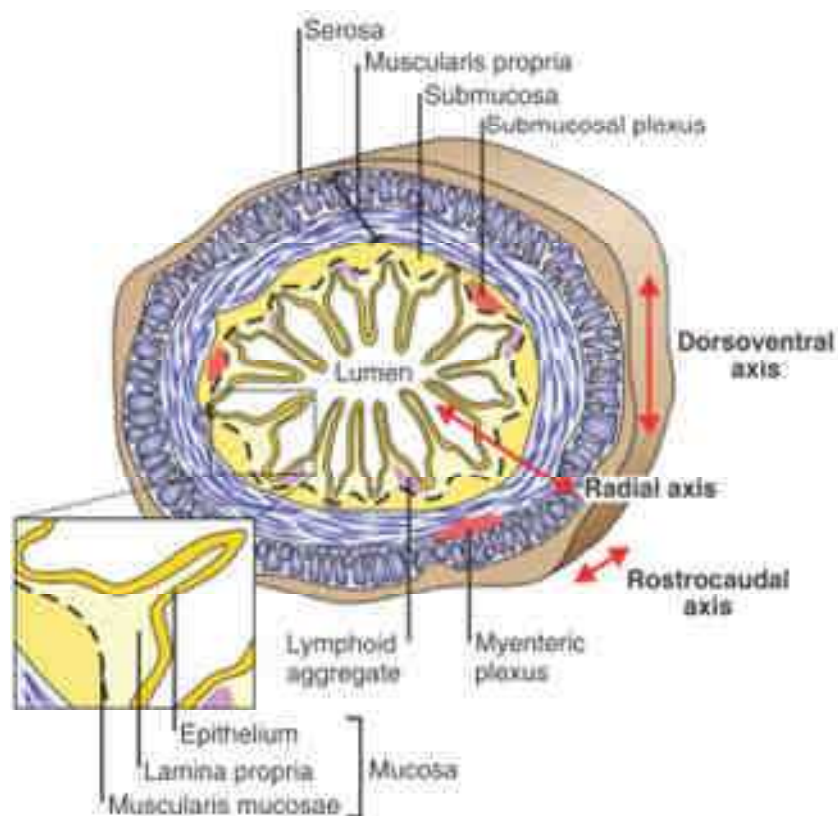


Figure 1: The multilayered structure of the intestine

The intestine can be depicted as a multilayered tube whose central cavity is called lumen. The different layers lining the intestine are: the *mucosa* (intestinal epithelium facing the lumen and lined by the loose connective tissue of the *lamina propria*), the *muscularis mucosa* (thin layer of smooth muscle), the *sub-mucosa* (contains nerves, blood vessels and lymphatic vessels), the *muscularis propria* (circular and longitudinal smooth muscles) and finally the *serosa* (Heath, 2010; Leon and Gregoriol, 2001).

The epithelium of the **small intestine** is composed of invaginations, the crypts of Lieberkühn, and finger-like protrusions, the villi, increasing the absorptive surface. The stem cells and the proliferating cells reside in the crypts, whereas differentiated cells are lining the villi (**Figure 2A**). The mucosa of the **colon** is a flat surface epithelium without any villi but only crypts. The upper third of these crypts is composed of differentiated cells and the bottom two-thirds is occupied by proliferating cells (**Figure 2B**). The intestinal epithelium is continuously renewing, with most of the cells moving up the crypt as differentiation proceeds. As they reach the uppermost part of the epithelium, they undergo cell death and are shed away into the lumen. This implicates that most intestinal cells have a very short life span of only 5-6 days. With the stem cells, the Paneth cells, which reside at the crypt bottom of the small intestine after differentiation, are the only ones which have a longer life span as they survive up to 3-6 weeks (Barker et al., 2008; Radtke and Clevers, 2005).

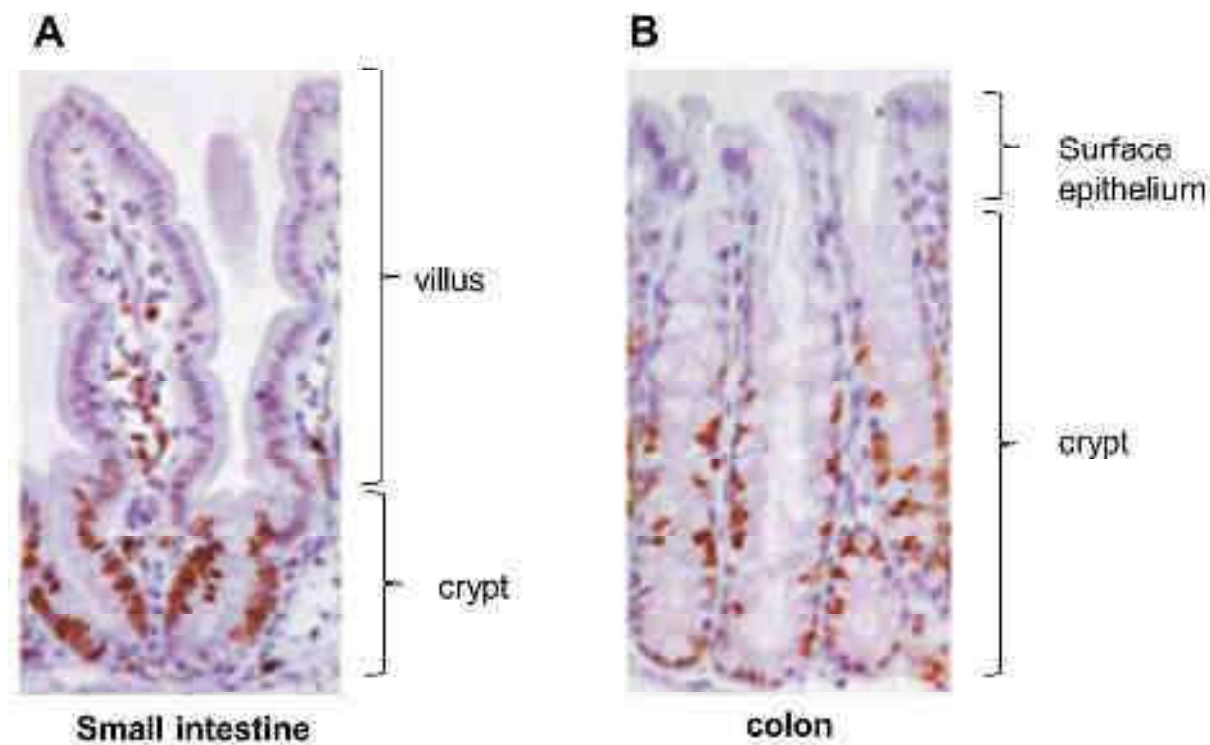


Figure 2: Structure of the intestinal epithelium

Shown are histological sections of the murine epithelium in the small intestine (**A**) and colon (**B**). Proliferative cells are stained with Ki67 (Radtke and Clevers, 2005).

2. Cellular aspects of the intestinal epithelium

2.1. Intestinal Stem cells

Because of the harsh environment, intestinal cells have a very short life span. To deliver enough cells for this continuously renewing epithelium, the crypt progenitors divide constantly and generate about 250 cells per crypt every day that will populate the surrounding villi. The constant production of new cells is ensured by stem cells that reside deep in the crypt, where they give rise to the highly proliferative transient amplifying cells (TA). These stem cells are defined by their capacity for self-renewal and for multipotency (i.e. the potential to generate all the differentiated cells of the tissue of origin). However, the precise identity / location of these cells and how exactly they divide is still intensely debated (Barker et al., 2010). The results obtained until today indicate that there are at least two functional groups of stem cells in the intestine.

The first type of intestinal stem cells was already described in the 1950's by Chris Potten, who observed that just over the Paneth cells (position +4) reside cells that were DNA label-retaining and resistant to radiation (Yan et al., 2012). However, there was no proof that these cells could generate all the cell types of the intestine until lineage tracing was performed in mice models using the potential stem cell marker Bmi1. In Bmi1^{Cre-ER/+} Rosa26^{LacZ/+} mice, it was shown that Bmi1 positive cells were self-renewing and gave rise to all intestinal cell types. The ablation of Bmi1 expressing cells induced cell death and loss of the crypt. However, since the number of Bmi1 positive cells decreases from the duodenum to the colon where no such cells are detected, other stem cell markers will have to be identified for the +4 position cells in the distal small intestine and the colon (Sangiorgi and Capecchi, 2008) (**Figure 3A, B**).

A second type of stem cells was described in the bottom of the crypt. These slender, immature, cycling cells, are called CBCC (Crypt Base Columnar cells) and are intercalated between the Paneth cells. As they are long lived, they were proposed to function as intestinal stem cells. A marker of CBC cells is the orphan receptor Lgr5 (leucine-rich G protein-coupled receptor 5), as its expression was detected exclusively in these cells. Lineage tracing experiments showed that Lgr5+ cells were capable of generating all cell types of an intestinal epithelium (Barker et al., 2007). An additional proof for the stem cell function of Lgr5+ cells came from *in vitro* experiments, in which single Lgr5+ cells were able to form organoids (crypt-like structures, containing enterocytes, goblet cells, enteroendocrine cells and Paneth cells) (Sato et al., 2009). Further studies found other potential markers for this cell population, such as Prom1/CD133 (Zhu et al., 2009), Ascl2 and Olfm4 (van der Flier et al., 2009) (**Figure 3C, D**).

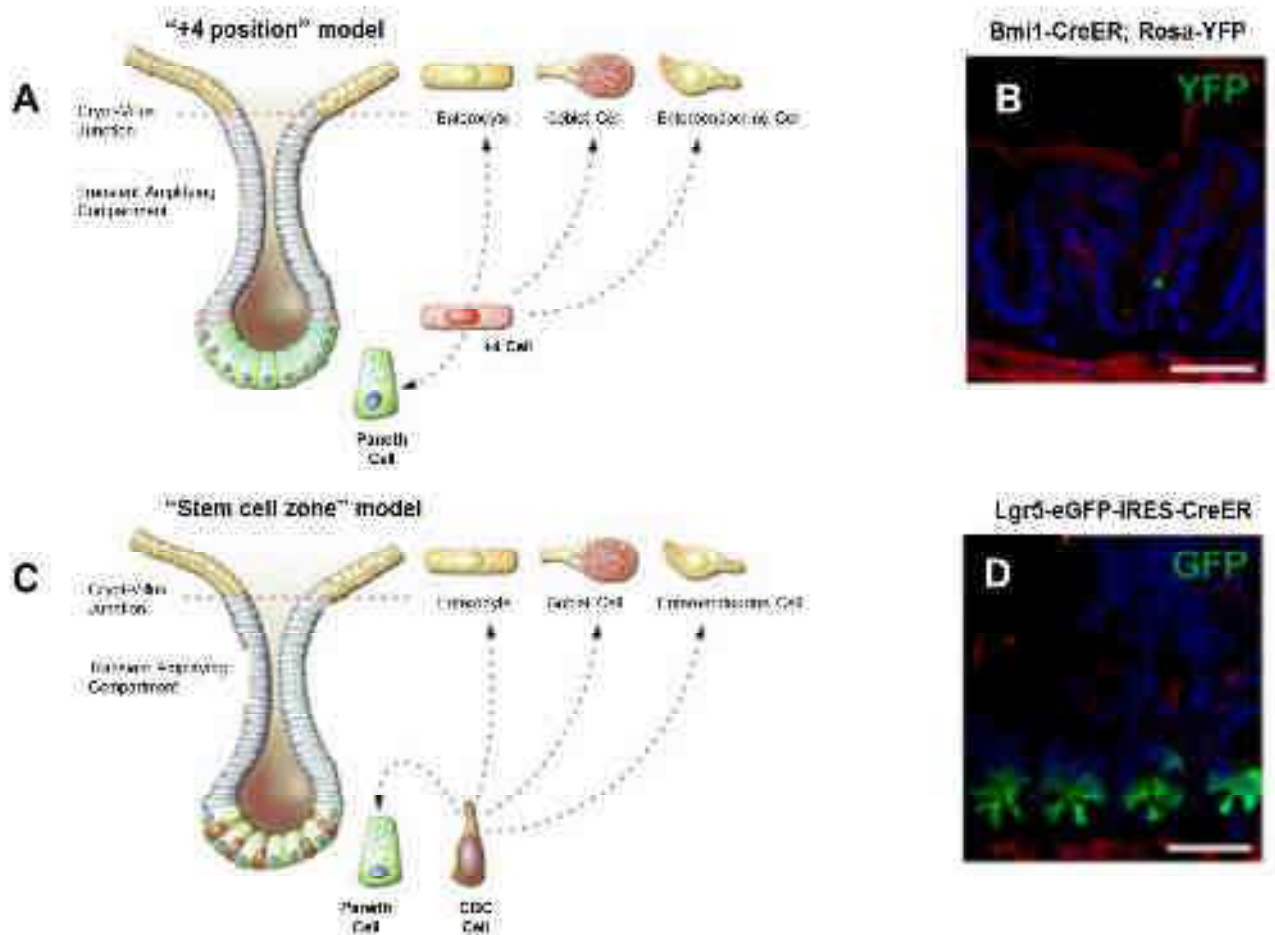


Figure 3 : Two stem cell models in the intestine

Schematic drawing of the +4 position model (A) and the stem cell zone model with CBC cells (C). Also shown are the localization of the +4 cells in Bmi1-CreER/Rosa26-YFP mice (B) and of the CBC cells in Lgr5-eGFP-IRES-CreERT2 (D) (Barker et al., 2008; Yan et al., 2011).

Interestingly, it was recently reported that after ablation of Lgr5+ cells, the number of Bmi1+ cells increased and that they restored the Lgr5+ stem cell compartment. This result indicates that the two stem cell populations are closely interrelated, but might have different roles. The CBC cells are actively dividing and would correspond to the 'workhorse stem cells' fuelling the daily self-renewal of the small intestine. The smaller pool of slow cycling +4 position cells would in contrast function as a stem cell reserve for times of crisis (Tian et al., 2011; Yan et al., 2011).

2.2. Differentiated cell types of the intestinal epithelium

Two main cell lineages constitute the intestinal epithelium, the absorptive lineage (enterocytes/colonocytes) and the secretory one (goblet cells, enteroendocrine cells, Paneth cells and Tuft cells). These cells are arranged to form a simple and columnar epithelium, separated by a basement membrane from the lamina propria, a loose connective tissue containing mesenchymal cells and immune cells (Figure 4).

Enterocytes: These absorptive cells correspond to the main cell type in the small intestine (> 80%). In the colon, the colonocytes are the absorptive cells and represent about half of the cells. Enterocytes secrete digestive enzymes, absorb nutrients from the lumen and release them on their basal cell surface. Their apical side is covered by microvilli, forming the so-called brush border, and maximizing the surface of absorption. This kind of cellular specialization is typical of epithelia with high absorptive or secretory activity, like those of the intestine or the kidney, and is characterized by its F-actin-based cytoskeletal core. In the intestine, digestive enzymes are anchored into the apical plasma membrane of microvilli as integral membrane proteins and it seems that some are even localized in specialized detergent resistant membrane regions (lipid rafts). Membranous transporter proteins will then allow absorption of the digested nutrients (Danielsen and Hansen, 2006).

Goblet cells: These mucus-secreting cells are found in the whole intestinal epithelium, but are the most abundant in the jejunum and colon. They produce the mucus that covers the epithelium and protects cells from mechanic stress and pathogens.

Enteroendocrine cells: They represent less than 1% of the intestinal cells and secrete hormones (Ex: motilin, serotonin, substance P, secretin...) that reach the blood vessels through the underlying lamina propria and control important physiological functions of the digestive system.

Paneth cells: They reside at the crypt base and are not present in the colon. They secrete lysozyme and antimicrobial peptides, such as defensins, into the lumen of the small intestine and thereby are implicated in the antibacterial host defense of the gut (**Figure 4**). Recent results indicate that Paneth cells could play an additional important role in maintaining the stem cell niche, as the presence of Paneth cells in cell culture strongly improved the formation of organoids from single cells (Sato et al., 2011). However, the absolute requirement of Paneth cells in establishing/maintaining a functional stem cell compartment *in vivo* remains controversial (Sato et al., 2011; Kim et al., 2012).

Other rare cell types that are found in the intestinal epithelium are for instance **M cells** (located in the epithelium overlying Peyer's patches and involved in mucosal immunity) and **Tuft cells** (characterized by thick long microvilli on their apical surface and secretion of opioids into the intestinal lumen) (Sancho et al., 2004; Radtke and Clevers, 2005; Crosnier et al., 2006; Noah et al., 2011).

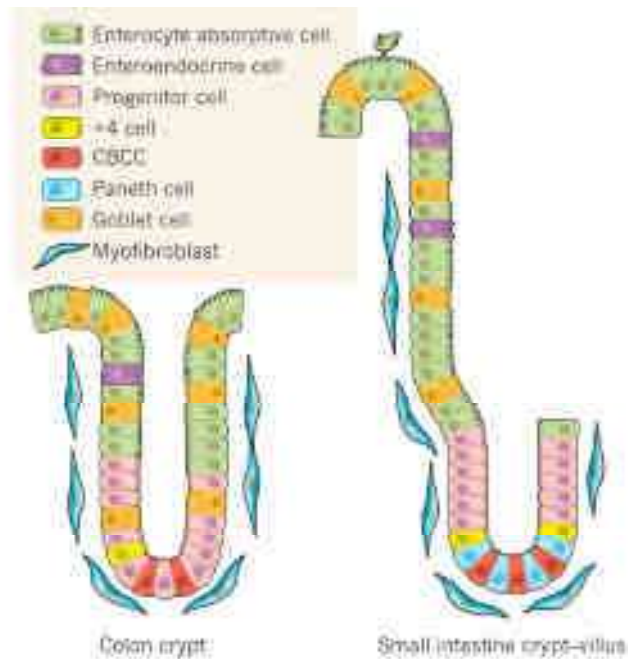


Figure 4: Localization of cell types in the small intestine and colon

Different cell types compose the intestinal epithelium. In the crypts are found undifferentiated stem cells (+4 cells, CBCC) and progenitors or transient amplifying (TA) cells, whereas in the villi reside differentiated enterocytes, enteroendocrine cells and goblet cells. Paneth cells are the only differentiated cells found at the crypt base. The intestinal epithelium is separated by the basement membrane from the lamina propria which comprises mesenchymal cells (Ex: myofibroblasts) (Medema and Vermeulen, 2011).

2.3. Cellular features of the epithelium that are critical for intestinal homeostasis

Integrity and proper functioning of the intestinal epithelium are ensured by a combination of morphological and functional features, some of them being especially remarkable.

- *Highly specialized cells*: progenitors differentiate into very particular cells dedicated to specific functions. These cells have distinct morphological traits (Ex: microvilli, secretory granules) and express different sets of genes (Ex: enzymes, transporters, hormones, antibacterial peptides).

- *Sophisticated intercellular adhesion mechanisms*: to prevent luminal pathogens and harmful substances from entering the internal milieu (barrier function) and yet promote digestion and absorption of nutrients, intestinal cells are tightly connected to each other by junctional complexes at the plasma membrane (namely tight junctions and cadherin-based adherens junctions, see IV) page 54). These junctions are connected to the intracellular cytoskeleton and contribute to the columnar cell shape.

- *Strong apico-basal cellular polarity*: as intestinal cells are in contact and interact with different environments, they are organized in morphologically (Ex: apical microvilli) and functionally (Ex: apical transporters and digestive enzymes of the brush border, basal transporters and solute carrier proteins) distinct domains. Junctional complexes also exhibit a polar distribution (Ex: apical tight junctions) and might be at the origin of polarity (Yeaman et al., 1999).

3. Signaling pathways regulating intestinal homeostasis

The intestinal epithelium is continuously renewing, as it reforms every 5-6 days. To maintain the complex architecture of the villi and crypts, several signaling pathways functionally interact to regulate the balance between proliferation, cell fate decision, cell sorting and differentiation.

3.1. The Wnt pathway

In mammals, multiple Wnts exhibiting diverse expression profiles and playing a variety of important roles in cell differentiation, proliferation and cell polarity, have been identified. These secreted glycoproteins bind to cell surface receptors and thereby activate three different pathways, depending on the secreted Wnt subtype: the canonical Wnt/ β -catenin cascade and the two non-canonical Wnt pathways, i.e. the planar cell polarity (PCP) pathway and the Wnt/ Ca^{2+} pathway (Gregorieff and Clevers, 2005). In the intestine, Wnts activating the canonical Wnt pathway are secreted by the crypt epithelial cells (Gregorieff and Clevers, 2005).

At the moment, the canonical Wnt pathway is the best understood and β -catenin plays a central role in its signal transduction, whereas the other two pathways are β -catenin independent. Few information about the role of non-canonical Wnt pathways in the intestine is available, however it has been shown that Wnt5a, an activator of the non-canonical Wnt pathway, is involved in the elongation of the intestine during embryonic development (Cervantes et al., 2009). Secretion of Wnt5a by intestinal myofibroblasts can also induce the expression of Cdx2, an important transcription factor inducing intestinal differentiation (Pacheco and Macleod, 2008). In contrast, it is well established that the canonical Wnt pathway is critical for several processes in the intestine, including maintenance of stem cells, proliferation of crypt progenitors, differentiation of Paneth cells and sorting of the differentiated cells along the crypto-villus axis (van der Flier and Clevers, 2009; Gregorieff and Clevers, 2005; Noah et al., 2011).

In the canonical Wnt pathway, upon absence of Wnt signaling, β -catenin is associated with E-cadherin at the cell membrane (**Figure 5A**). Cytoplasmic β -catenin is phosphorylated in a destruction complex scaffolded by Axin and APC. The first phosphorylation occurs at Ser45 by casein kinase 1 (CK1), followed by phosphorylations of Thr41, Ser 37 and Ser33, by glycogen synthase kinase 3 (GSK3). This labels β -catenin for ubiquitination by a beta-TrCP-containing E3 ubiquitin ligase, followed by the degradation in the proteasome.

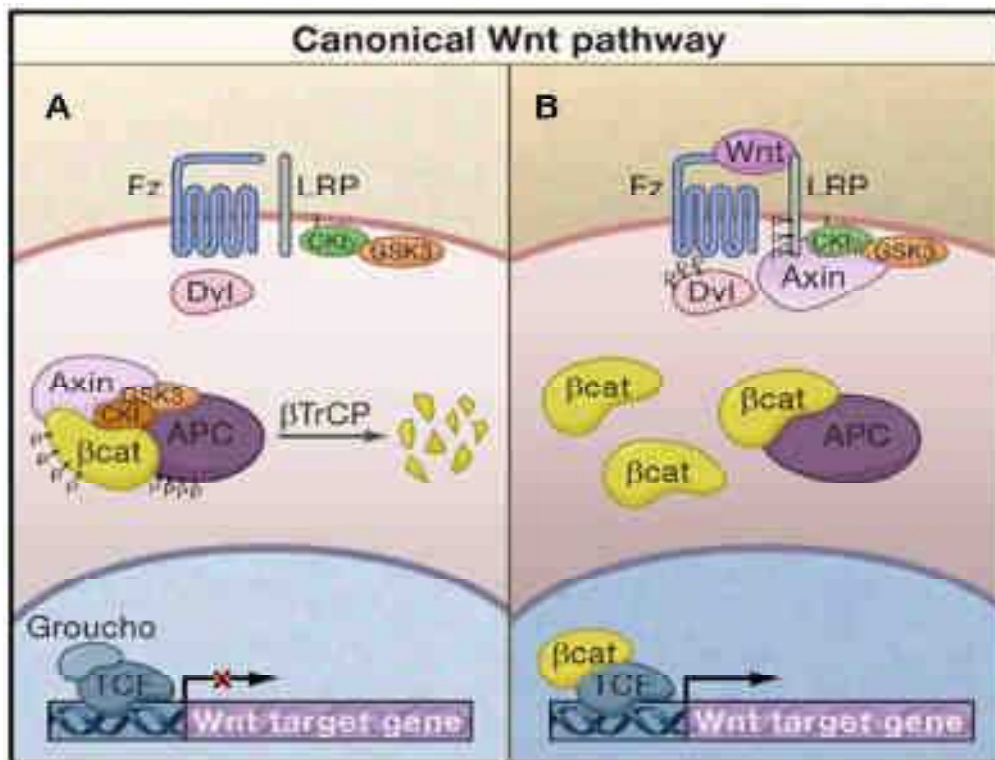


Figure 5: The canonical Wnt pathway

(A) In the absence of Wnt, β -catenin is degraded in the proteasome after phosphorylation in a destruction complex containing Axin, APC, GSK3 and CK1. **(B)** Upon Wnt binding to Frizzled and its co-receptor LRP, the destruction complex is inhibited and β -catenin translocates to the nucleus, binds to TCF and activates TCF target gene expression (Gregorieff and Clevers, 2005).

The canonical Wnt pathway is activated by diffusible Wnt proteins, secreted by the epithelial cells, that bind to seven-pass transmembrane receptors Frizzled (FZ) associated to the co-receptor LDL receptor-related proteins 5 and 6 (LRP5 or LRP6) in the intestine (**Figure 5B**). This activates Dsh (Dishevelled) that recruits Axin to the cell membrane, thereby inhibiting the destruction complex. In consequence, β -catenin escapes degradation, accumulates in the cytoplasm and is finally translocated to the nucleus. In the absence of β -catenin, TCF/LEF transcription factors are associated with the co-repressor Groucho and inhibit gene transcription. Nuclear β -catenin displaces Groucho, forms a complex with TCF (T cell factor) and activates target gene transcription (Gregorieff and Clevers, 2005).

The activity of the canonical Wnt pathway can be modulated at different levels. Extracellular ligands can act as agonists of Wnt signaling (Ex: R-spondin) or inhibitors of Wnt signaling (Ex: Dickkopf 1). Inside the cells, the activity of β -catenin is not only regulated by its stability, but also fine-tuned by multiple post-transcriptional modifications and interactions with other molecules. For example, the nuclear concentration of β -catenin is affected by APC, which shuttles β -catenin out of the nucleus, or Bcl9, which retains β -catenin in the nucleus (Gregorieff and Clevers, 2005). Moreover, PKA and Akt phosphorylations on Ser552 and Ser675 of β -catenin can enhance β -catenin/TCF/LEF transcriptional activity (Daugherty and Gottardi, 2007).

In the transcription process, β -catenin can also interact with different co-activators that contribute to cell-specific gene expression (Rao and Kühl, 2010; Brabletz et al., 2005a).

The canonical Wnt pathway is essential for the maintenance of the stem cell niche and the transient amplifying compartment: consequently, Wnt target genes are implicated in stem cell formation (Ex: Survivin, Lgr5) (Barker et al., 2007; Zhang et al., 2001) and cell cycle progression (Ex: c-Myc, Cyclin D1). Other target genes are implicated in cell differentiation of Paneth cells (Sox9) (Noah et al., 2011) and cell sorting (Ex: EphB-2 and -3) (Sansom et al., 2004). Furthermore, some Wnt target genes are implicated in proteolysis and cell migration (Ex: MMP-7, Slug, CD44) (Brabletz et al., 2005a; Barker et al., 2007), functions that are especially important during embryonic development. Of note, no universal target genes have been identified: transcriptional activation is cell type and context specific and depends on the concentration of β -catenin (Brabletz et al., 2005a; Clevers, 2006).

3.2. The Notch pathway

One of the first decisions when cells start to differentiate is whether they commit themselves to the secretory or absorptive cell lineage. The Notch pathway plays a key role in this cell fate decision by exerting lateral inhibition between cells.

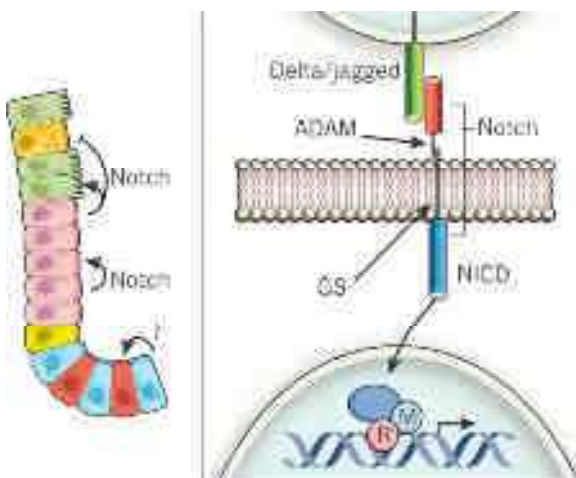


Figure 6: The Notch pathway

The Notch ligand and the Notch receptor are expressed on neighboring cells. Ligation of the Notch ligands Jagged/Delta induces the cleavage of the Notch receptor, and the intracellular domain NICD will translocate to the nucleus where it activates target gene transcription (Medema and Vermeulen, 2011).

The Notch receptor and its ligands from the Delta and Jagged families are transmembrane proteins located on neighbor cells in the intestinal crypt. Ligand binding will induce the double cleavage of Notch by ADAM5 (extracellular part) and γ -secretase (transmembrane domain), liberating the intracellular domain of Notch called NICD (Notch Intracellular Domain) (**Figure 6**). This activated fragment will translocate to the nucleus, where it binds to RBP-Jk (CSL or CBF1), recruits mastermind-like (MAML) adaptor proteins and activates gene expression. The best-characterized Notch target gene encodes the transcription factor Hes1 (Hairy/Enhancer of split) which on its turn inhibits Math1 (mouse homologue of Atonal) expression, a transcription factor required to initiate differentiation of all secretory cell types: thus, activation of the

Notch pathway in a progenitor will drive it towards the absorptive lineage. Moreover, Hes1 will inhibit the transcription of the Notch ligand Delta: thus, activation of the Notch pathway in a progenitor will also drive neighboring cells towards the secretory lineage (Radtke and Clevers, 2005).

3.3. The BMP pathway

BMPs (Bone Morphogenetic Proteins) are members of the TGF- β superfamily and transduce signals from the underlying stroma to the intestinal epithelium that are critical for both intestinal development and adult organ homeostasis. For instance, BMP signaling has been shown to control the architecture of the intestinal epithelium by overexpression of the BMP inhibitor Noggin in transgenic mice: the blocking of BMP signaling led to the appearance of crypts that were perpendicular to the crypt-villus axis everywhere in the epithelium (Haramis et al., 2004).

In the intestine, BMP-2 and BMP-4 are secreted by mesenchymal cells in the intervillus region and will act on differentiated epithelial cells, which express the BMP receptors and SMADs. Ligation of BMPs clusters two cell surface serine–threonine kinase receptors (type I and type II), which results in the phosphorylation of the type II receptor by the type I receptor. This induces the phosphorylation of receptor-regulated R-SMADs (1, 5 or 8), their heterodimerization with the co-SMAD SMAD4 and their translocation to the nucleus where they stimulate transcription of target genes like SMADs, Gata4, RUNX2 and the BMP own antagonist Noggin (**Figure 7**) (Zwijsen et al., 2003).

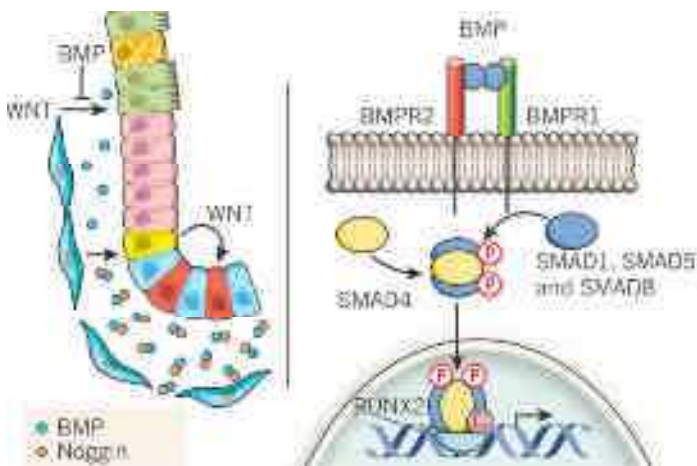


Figure 7: The BMP Signaling

BMP binding induces dimerization of the receptor kinase I and II. This will trigger phosphorylation of R-SMADs (SMAD -1, -5, or -8) that will bind in consequence to a co-SMAD (SMAD4). This complex translocates to the nucleus and activates target gene transcription (Medema and Vermeulen, 2011).

3.4. The Sonic Hedgehog pathway

Another pathway that implicates epithelial/mesenchymal interaction is the Sonic Hedgehog (SHH) pathway, but in opposition to the BMP pathway, the epithelium is signaling to the mesenchyme in the SHH pathway. Indeed, the ligand Shh (Sonic hedgehog) is thought to be expressed by the +4 stem cells, whereas the ligand Ihh (Indian hedgehog) is secreted by the differentiated epithelial cells. The Hedgehog signaling receptors Patched1 and Patched2 (PTCH1, PTCH2) and their downstream effectors Gli1, Gli2 and Gli3 are expressed in mesenchymal cells (**Figure 8**).

Hedgehog signaling between the epithelium and the mesenchyme is required for both villi formation and the delimitation of proliferation to the intervillus regions. Indeed, inhibition of Hedgehog signaling in mice led to the absence of villi and cell differentiation, which correlated with increased Wnt signaling activity (Crosnier et al., 2006).

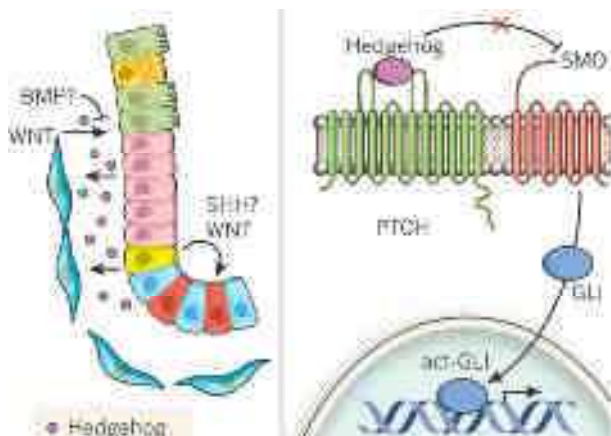


Figure 8 : The Hedgehog pathway

Shh is secreted by epithelial cells and binds to the PTCH receptors on mesenchymal cells. The blocking of SMO by PTCH is inhibited by binding of the hedgehog ligand leading to activation of the transcription factor Gli (Medema and Vermeulen, 2011).

3.5. Coordinated interactions of the various signaling pathways

All these signaling pathways are crucial for the formation of the stem cell niche, crypt-villus architecture, and cell differentiation / sorting. As these are very complex processes that need to be coordinately regulated, the different signaling pathways reciprocally interact with each other to shape and maintain the intestinal epithelium.

At the crypt base, the maintenance of stem cells requires cooperation of the Wnt and the Notch pathways (Crosnier et al., 2006; Medema and Vermeulen, 2011). Similarly, cell fate decision between the absorptive and secretory lineages involves coordinated modulation of the Wnt and the Notch pathways, namely by Nox1 (Coant et al., 2010).

In contrast, the development of intestinal crypts implicates the Wnt and the BMP pathways that antagonize each other. Indeed, inhibition of Wnt signaling by forced expression of the diffusible Wnt inhibitor Dkk1 or by knocking out TCF, results in a loss of crypts and reduction of villi size in mice (Gregorieff and Clevers, 2005). The BMP pathway opposes the action of the Wnt signaling via the tumor suppressor PTEN, thereby counterbalancing its effect on differentiation and proliferation (He et al., 2004) and blocking crypt development. Therefore, secretion of the diffusible BMP repressor Noggin by the stroma surrounding the crypt area permits Wnt signaling and crypt formation (Crosnier et al., 2006). Moreover, it seems that Indian hedgehog (IHH) signals to the mesenchyme, where it is thought to induce BMP secretion, thereby counteracting Wnt signaling (Medema and Vermeulen, 2011). Of note, this illustrates how signals from the micro-environment of the crypts (BMP, Noggin) and epithelial factors (Wnts, SMADs, IHH) act together in maintaining tissue homeostasis.

Cell sorting along the crypt-villus axis is also controlled by the Wnt pathway, through its action on Ephrins, which are responsible for the correct positioning of proliferative and differentiated cells: the tyrosine kinase guidance receptors EphB-2 and -3 are TCF target genes expressed in the crypt, whereas the transmembrane ligand Ephrin-B1, whose expression is repressed by Wnt, is detected in villus cells and repulses cells expressing Eph-B2 or Eph-B3, preventing the proliferative cells to leave the crypt (Crosnier et al., 2006).

These examples only give a slight insight into the complexity of the intestinal homeostasis, and several other pathways (Ras, PI3K/Akt...) are necessary to maintain a functional epithelium. Even if significant progress has been achieved in the last years in the understanding of the mechanisms involved in the maintenance of the stem cell niche and the differentiation processes, many questions remain to be answered (Crosnier et al., 2006; Noah et al., 2011).

In summary, the small intestinal epithelium is organized in functional compartments, i.e. the crypts and villi. Crypts are harboring stem cells and highly proliferative transient amplifying cells, whereas the villi are lined with differentiated cells that assure the function of the intestinal epithelium (nutrient absorption, mucus production...). Although the colon has no villi, it presents a similar functional organization. The cells of the intestinal epithelium are continuously renewed and many signaling pathways act together to establish the crypt villus architecture and to coordinate proliferation and differentiation. Deregulation of the different processes (aberrant proliferation, defects in cell differentiation, decreased cell adhesion) can lead to intestinal pathologies, namely colorectal cancer.

II) Colorectal Cancer (CRC)

1. General information on colorectal cancer

1.1. Epidemiology

In 2008, statistical estimations ranked colorectal cancer as the most common cancer in Europe with 435,600 cases per year and as the second cause for cancer death (212,000 deaths/year) after lung cancer. In recent years, mortality rates have generally declined, although the incidence of CRC has modestly increased in most European countries (Ferlay et al., 2009). Overall, the European 5-year relative survival rate for CRC is about 50% and most of CRC deaths are due to metastases (Ballinger and Anggiansah, 2007).

1.2. Risk factors

In Western countries, the lifetime risk of developing CRC is \approx 5% but the following factors are associated with an increased risk.

- Age: 85% of diagnosed patients are more than 60 years old (Ballinger and Anggiansah, 2007).
- Lifestyle: the consumption of red meat, obesity and a sedentary lifestyle seem to enhance the development of CRC. Accordingly, incidence of CRC is increasing in Asia, as western lifestyle becomes more popular in these countries (Markowitz et al., 2002; Ballinger and Anggiansah, 2007).
- Inflammatory bowel diseases: chronic inflammation in ulcerative colitis and Crohn's disease patients increases the risk to develop CRC (Ballinger and Anggiansah, 2007). This is in line with the observation that daily intake of anti-inflammatory aspirin can reduce the risk for colorectal cancer (Thun et al., 2012).
- Familial history: most of CRC are sporadic, but \approx 20% of patients have a family history of the disease (Hollande et al., 2010). Indeed, first-degree relatives of CRC patients have about twice the risk of developing the disease compared to individuals with no family history. This increased incidence might be linked to hereditary (genetic alterations), lifestyle or environmental factors (Ballinger and Anggiansah, 2007).

For a few inherited forms of colon cancer, the genes implicated are identified. **Familial Adenomatous Polyposis** (FAP, 1% of CRC) patients share a germline mutation in the colonic tumor suppressor gene APC. The somatic mutation of the second APC allele induces the formation of hundreds of adenomas (polyps) in the colon that can progress to adenocarcinomas, warranting a 100% lifetime risk of CRC without treatment (Markowitz et al., 2002). **Hereditary non-polyposis**

colon cancer (HNPCC or Lynch syndrome; 3% of CRC) patients have a germline mutation of DNA mismatch repair genes hMLSH1, hMLSH2 or hMSH6 that augments microsatellite instability and they have an increased lifetime risk to develop CRC (Markowitz et al., 2002). Rare hamartomatous polyposis conditions like **Juvenile polyposis** and the **Peutz-Jeghers** syndromes also increase the risk to develop CRC (Markowitz et al., 2002).

1.3. Clinical and pathological classification of CRC

The stage of a tumor describes its spread through the body. It is an important predictor of patient survival and mostly determines treatment. Staging combines information obtained through physical examination, radiology, endoscopy and microscopy analysis of biopsies. The **TNM** tumor stage informs about the local extension of the tumor (**T**), the presence and number of lymph node metastases (**N**) and of distant metastases (**M**), which most often occur in liver and lung (**Figure 9**) (Markowitz et al., 2002). A colon tumor is considered malignant when cells cross the muscularis mucosa and invade the sub-mucosa since metastasis can only occur after this step. Indeed, this layer harbors the lymphatic and blood vessels that tumor cells use to reach distant sites (Leon and Gregoriol, 2001).

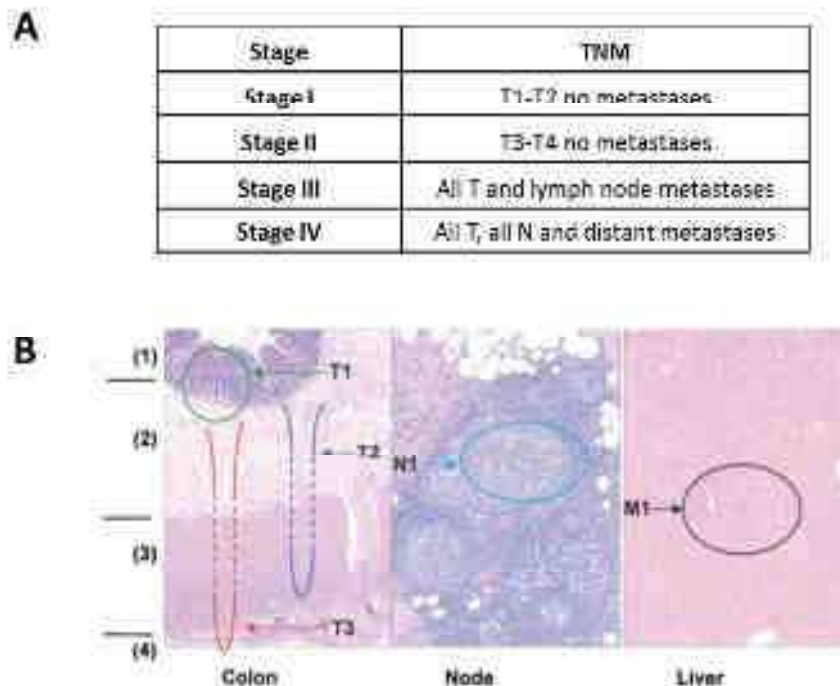


Figure 9: Definition of tumor stages with the TNM staging system

(A) Table detailing the different stages and the corresponding TNM properties. **(B)** The left image shows the principle layers of the colon including the mucosa (1), the sub-mucosa (2), the muscularis propria (3), and the sub-serosa (4). T1 - tumors cells have not crossed the muscularis mucosa, T2 – cells invaded the sub-mucosa, T3 – cells entered the muscularis propria, T4 – invasion into the serosa or adjacent structures. The middle and right images show respectively lymph node and liver metastases (Markowitz et al., 2002).

CRCs are also characterized according to their **grade**. It refers to the overall differentiation status of the primary tumor. Low grade tumors appear very similar to normal tissue, whereas high grade tumors are poorly differentiated. Patients presenting low grade tumors have a better prognosis than the ones with high grade tumors (Brabletz et al., 2005a).

1.4. Prognosis and treatment of CRC

The clinical, pathological and molecular characteristics of the tumor determine its prognosis and treatment which mostly depend on the stage at the moment of the diagnosis (**Table 1**). The prognosis for patients diagnosed at stage I or II is quite good, however 20-25% of patients have already metastases at the moment of diagnosis which dramatically decreases their 5-year survival rate to 8% (Pozzo et al., 2008; Hollande et al., 2010) as metastases are the major cause of death in CRC patients (Bird et al., 2006). Thus, early detection of CRC by systematic screening (occult blood stool detection, colonoscopy) may significantly decrease the mortality associated with this cancer.

| Stage at Diagnosis | 5-year survival |
|--------------------|-----------------|
| Stage I | 90% |
| Stage II | 75% |
| Stage III | 67% |
| Stage IV | 8% |

Table 1 : The 5-year survival of CRC patients depends on the tumor stage at diagnosis

(Hollande et al., 2010)

Surgery is the main treatment for CRC and is applied at all stages. For stage I cases, the resection of the tumor is in most cases sufficient to eradicate the cancer, whereas for stage II-IV cases adjuvant, neo-adjuvant or palliative chemotherapy is also administrated. Currently, the first line chemotherapies are based on combinations of cytotoxic drugs, which revealed to be more effective than a single anti-cancer drug. The FOLFOX regimen (combination of folinic acid, 5-fluouracil and oxaliplatin) is generally prescribed to stage II/III tumors, whereas the FOLFIRI regimen (combination of folinic acid, 5-fluouracil and the topoisomerase I inhibitor irinotecan) is recommended for stage IV tumors (O’Neil and Goldberg, 2008). Targeted therapy, using humanized monoclonal antibodies, is sometimes used in combination with cytotoxic drugs to treat metastatic colon cancer: Bevacizumab (Avastin™) inhibits angiogenesis by targeting the VEGF receptor and Cetuximab (Erbix™) blocks the EGF receptor, an enhancer of cell growth and survival (Hollande et al., 2010). Up to now however, targeted therapy for CRC seems to offer a slight benefit only for some patients (Ex: mutation status of

K-Ras for Cetuximab) and for a limited amount of time (development of resistance) (Misale et al., 2012).

2. Initiation and progression of CRC

2.1. Anatomic-pathological aspects

Over 90% of CRCs are adenocarcinomas. They generally develop from benign lesions called adenomas (or adenomatous polyps), which correspond to well-demarcated epithelial dysplasia with uncontrolled proliferation. Only a small portion of adenomas evolves into an adenocarcinoma with a malignant phenotype. Adenocarcinomas present a very heterogeneous pattern on the histological level. This heterogeneity is observed between tumors from different patients but also within the same tumor: indeed, cell populations that vary in their morphology and behavior (differentiation, proliferation, invasiveness) co-exist in a tumor. In general, the tumor center harbors more differentiated areas, whereas the cells at the tumor periphery are poorly differentiated and show invasive behavior (Brabletz et al., 2005a). The presence of invasive cells at the tumor edge or disseminated in the adjacent stroma is associated with metastasis formation and consequently with poor prognosis (Spaderna et al., 2006). Tumor cells able to cross the basal membrane and the muscularis mucosa will reach the sub-mucosa which is a prerequisite to form metastases (Leon and Gregoriol, 2001).

The crucial step in carcinogenesis is the invasion of adjacent tissues by the tumor cells and formation of distant metastases. In CRC, tumor cells will mostly disseminate to the lymph nodes through the lymph vessels and to the liver as the portal vein conducts blood from the gastrointestinal tract directly to this organ. Less often, distant metastases are observed in the lungs, ovaries, peritoneum and brain. During the metastatic process, the tumor cells detach from the primary tumor mass, invade neighboring tissues, enter into the blood stream (intravasation), survive in the circulatory system, leave the blood vessel (extravasation) and form a secondary tumor in a foreign tissue. It is interesting to note that the metastasis generally recapitulates the histologic organization of the primary tumor, with differentiated areas in the tumor center and invasive cells at the host-tumor front (Brabletz et al., 2005a, 2005b).

2.2. The “Fearon and Vogelstein” model

It is generally considered that carcinogenesis is initiated and driven by the stepwise accumulation of genomic alterations. Fearon and Vogelstein proposed such a model of progression for CRC: they performed genomic analysis of CIN tumors at different stages of the adenoma-carcinoma sequence and found that certain mutations occurred at a high frequency at important phases of tumor development (Fearon and Vogelstein, 1990).

According to the Fearon and Vogelstein model, alterations of genes implicated in the Wnt pathway are initiating events in the development of CIN tumors. This correlates with the observation that the majority of CRC patients show sporadic somatic mutations in APC (over 80%) or other genes implicated in the Wnt pathway, such as β -catenin or Axin2 (Radtke and Clevers, 2005). APC is a classical tumor suppressor whose inactivation in mice results in tumor formation (Su et al., 1992) and FAP patients harboring a germline mutation of APC are sure to develop colon cancer by the age of 40 without clinical care (Markowitz et al., 2002). Loss of APC impacts on proliferation and stemness maintenance through the Wnt/ β -catenin signaling pathway, but also on other processes such as cell adhesion and migration, organization of the actin and microtubule networks, spindle formation and chromosomal segregation, all potentially involved in tumor initiation and progression (Aoki and Taketo, 2007).

Later in tumor progression, other tumor suppressor genes or oncogenes are frequently altered: mutations of K-Ras and p53 occur in 50% and of the TGF-beta receptor II in 30% of colon adenomas (**Figure 10**) (Markowitz et al., 2002). Of note, these genes are also often altered in MIN tumors (Markowitz et al., 2002; Gerstung et al., 2011; Grady and Carethers, 2008).

Nevertheless, it seems nowadays obvious that the Fearon and Vogelstein model of tumor progression is a little bit simplistic. Indeed, **colon tumors exhibit a high level of molecular heterogeneity**. Different tumors exhibit variable genomic alterations and the degree or type of alteration does not necessarily correlate with the tumor stage (Weber et al., 2007). Even within the same tumor, cancer cells exhibit various genomic alterations (Goranova et al., 2011). In addition, no genomic alteration could be associated with the most critical step of tumor progression, i.e. metastasis (Jones et al., 2008).

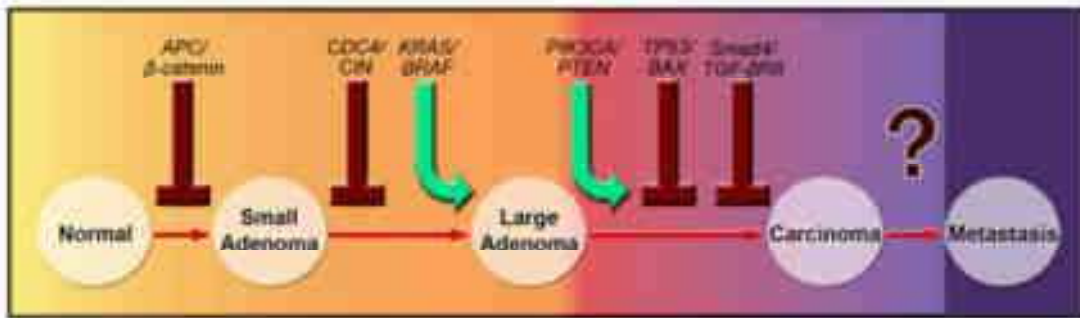


Figure 10 : Mutations driving tumor progression in CIN tumors

According to the Fearon and Vogelstein model, the loss of tumor suppressors (APC, SMAD4) and the gain of oncogene activity (β -catenin, K-ras) with a high frequency and in an ordered manner enables tumor progression (Jones et al., 2008).

2.3. The cancer stem cell model

More recently, the discovery of cancer stem cells (CSC) within solid tumors has changed our view of carcinogenesis. In opposition with a model in which random (stochastic) mutations are selected through clonal selection, a hierarchical model postulates that only a minority of tumor cells, i.e. the CSC, would be able to initiate and maintain the tumor (Simons and Clevers, 2011). Similarly to normal stem cells, these CSC are defined as self-renewing, long-lived and able to give rise to all the differentiated cells of the tumor (multipotency). Whether these CSC originated from altered adult stem cells or had just acquired properties of stem cells is unclear at the moment. Nevertheless, according to the CSC concept, the bulk of the tumor is composed of proliferating and differentiated post-mitotic cells, but is maintained by a small subset of **specific tumor cells with stem cell characteristics**, in a way that a tumor reflects the organization of a normal organ. The tumor initiating capacity and the multipotency of the CSC would also allow the formation of metastases very similar to the primary tumor. Given that the CSC seem more resistant to conventional treatments than the majority of the tumor cells, their presence might as well explain recurrence after elimination of the primary tumor by chemotherapy (Vermeulen et al., 2012).

For CRC and other cancers, one of the challenges resides in identifying the CSC, which is typically performed by xenografting limited amounts of specific tumor cells to immunodeficient mice. In the first studies on colorectal CSC, the transmembrane protein CD133 was proposed as a CSC marker, mainly because low amounts of CD133⁺ cells could initiate a tumor while CD133⁻ cells could not. However, several observations casted some doubts about its robustness as a colon CSC marker, namely its ubiquitous expression in the differentiated colonic epithelium (Simons and Clevers, 2011), but this might actually depend on the antibody used (Kemper et al., 2010). Other studies have suggested alternative colon CSC markers such as EpCAM, CD166 and CD44. As none of these markers

is exclusively expressed in stem cells, a combination of multiple markers might more reliably identify CSC (Zeki et al., 2011). Furthermore, recent studies highlighted the importance of the environment (the so-called stem cell niche) in the maintenance of the properties of normal stem cells through interactions of the Wnt, Notch and BMP pathways. This suggests that CSC are not a static cell population but rather that the CSC phenotype is dynamic and sensitive to its micro-environment (Simons and Clevers, 2011). Thus, even if the CSC model harbors conceptual and clinical potential, further investigation is required to prove the existence of these cells, characterize their origin, role, properties and regulation.

2.4. Plasticity of tumor cells

2.4.1. Potential importance of plasticity in tumor progression

Presence of metastases is the most important prognostic factor in CRC, but the mechanisms leading to tumor formation at distant sites are still debated. To detach from the tumor mass, go through the basement membrane and the vessels, survive and finally establish a secondary tumor in a foreign organ, cancer cells need to exhibit a whole range of specific properties, i.e. not possessed by all tumor cells. This led to the idea that a particular set of genomic alterations would be required to complete the metastatic process. In the 90's, Fearon and Vogelstein were not able to find supplementary genomic alterations linked to metastasis. Later, some studies even described metastases exhibiting less genomic alterations than the matching primary tumor (Weber et al., 2007), suggesting that the type of alteration was more significant than its accumulation (Jones et al., 2008; Goranova et al., 2011). In the same line, extensive gene expression profiling of tumors was done to define a "metastatic signature" but results were highly variable and no signature has been validated as a diagnostic or prognostic tool applicable to routine clinical practice for CRC (Nadal et al., 2007).

Altogether, this led to the idea that the last steps of tumor progression might not be "cell-autonomous" and sole depend on irreversible genomic alterations (Brabletz et al., 2005b). Indeed, epigenetic or transcriptional/post-transcriptional mechanisms, conditioned both by the genetic background and the micro-environment of the tumor cells, might contribute to the metastatic process. Accordingly, **transient modifications of protein expression or activity were specifically observed in invasive colon cancers cells**, primary tumor and metastasis cells being very similar, but the lack of lineage tracing evidence hampers any definitive conclusion about reversibility (Brabletz et al., 2005b; Thiery et al., 2009). Nevertheless, the fact that some tumor cells exhibit a certain degree of plasticity, involving reversible molecular modifications and sub-sequent changes in cell

morphology and function, would help to explain tumor heterogeneity and metastatic dissemination.

2.4.2. The EMT process and invasion

In CRC, tumor cell plasticity involves transitions between epithelial and mesenchymal states that would allow the tumor cells to detach from the primary tumor and disseminate, as well as to form secondary tumor masses. Similar transitions occur in healthy cells and are important for tissue remodeling. For instance, Epithelial-Mesenchymal Transition (EMT) occurs during neural crest cell migration in the embryo or wound healing in the adult (Thiery et al., 2009). Ectopic activation of signaling pathways (Ex: TGF β , Notch or Wnt pathway) involved in physiological EMT in cancer cells is associated with abnormal expression of key transcription factors (Ex: Zeb1 in CRC, Twist1 in breast cancer (Spaderna et al., 2008; Singh et al., 2011; Li et al., 2012; Mani et al., 2008)) or miRNA (Ex: miR-200 for CRC, miR-661 or miR-155 for breast cancer (Brabletz and Brabletz, 2010; Vetter et al., 2010; Kong et al., 2008)).

The morphogenetic changes associated with EMT are consistent with tumor cell dissemination: indeed, the cell loses its epithelial features, its typical shape, apico-basal polarity and strong intercellular adhesion, in favor of a mesenchymal-like phenotype (loose adhesion, increased motility). Tumoral EMT has also been linked with increased resistance to apoptosis (Kajita et al., 2004). On the molecular level, hallmarks of EMT are the loss of E-cadherin and the appearance of mesenchymal markers such as N-cadherin and vimentin. β -catenin, which is mostly linked to E-cadherin at the membrane in cells of the tumor mass, accumulates in the nucleus where it can stimulate the transcription of several TCF target genes favoring survival (Ex: Survivin) and invasion (Ex: MMP7, uPA). Furthermore, the actin cytoskeleton is reorganized by Rho small GTPases to activate the motility machinery and metalloproteases (Ex: MMP7 in CRC) are secreted to allow invasion (Christofori, 2006). All these modifications induce a fibroblast-like morphology in invasive cells, which makes it difficult to discern them from other mesenchymal cells in the tumor stroma. However, invasive cells having undergone tumoral EMT were detected in CRC tissues by the presence of remaining epithelial markers, such as cytokeratin 20 (Christofori, 2006). Importantly, the presence of these cells has been associated with poor clinical outcome and metastasis formation (Thiery et al., 2009; Spaderna et al., 2006; Zlobec and Lugli, 2010).

Since metastases recapitulate the organization of the primary tumor, with cells from the primary and secondary tumors exhibiting molecular and morphological similarities, the reverse transition (MET, Mesenchyme-Epithelium Transition) may occur in the invasive cells once they colonize a lymph node or a distant organ, but the hypothesis of a dynamic process awaits definitive proof (Thiery et al., 2009; Brabletz et al., 2005b).

2.4.3. Origin of metastatic cells

An important yet unresolved question is the origin of the tumor cells exhibiting EMT and whether all tumor cells possess plasticity. The observation that tumoral EMT mostly occurred at the periphery of the tumor suggested a strong influence of the micro-environment, which was confirmed by *in vitro* studies. For instance, modifications of the type of extracellular matrix proteins or of growth factors, hypoxia or inflammation have been shown to induce EMT (**Figure 11A**) (Brabletz et al., 2004; Vermeulen et al., 2010; López-Novoa and Nieto, 2009). Thus, **when confronted with a different micro-environment, mature cells with genomic alterations would undergo EMT**, which might help them to disseminate, and would fall back through a MET to their epithelial phenotype once they find themselves in an appropriate micro-environment (**Figure 11B**) (Brabletz et al., 2005b; Thiery et al., 2009). Whether these cells exhibit specific genomic alterations is not clear at the moment (Jones et al., 2008; Weber et al., 2007), but is suggested by the existence of metastatic signatures (gene expression pattern) in the primary tumors (Nadal et al., 2007). Another possibility is that the CSC or early progenitors are the tumor cells that intrinsically possess the plasticity required to switch between the epithelial and the mesenchymal/stem-like phenotype. The switch would be dictated by the CSC niche: for instance, stromal myofibroblasts secrete hepatocyte growth factor (HGF) able to restore a CSC phenotype in more differentiated tumor cells from CRC or breast cancer (Medema and Vermeulen, 2011).

T. Brabletz also proposed that invasive cells represent a specific type of CSC, the migrating CSC, located pre-dominantly at the tumor–host interface and derived from stationary CSC through the acquisition of a transient EMT in addition to stemness: EMT would confer mobility and stemness would grant the capacity of tumor initiation and multipotency (Brabletz et al., 2005b). Actually, it was recently shown in breast cancer that EMT and stemness exhibit molecular and even functional similarities: accordingly, EMT would generate cancer cells with stem cell-like characteristics or reciprocally CSC would express EMT markers (Thiery et al., 2009; Mani et al., 2008). Anyhow, since both EMT and stemness have not only been associated with tumor dissemination, but also with increased resistance to apoptosis (Zeki et al., 2011; Kajita et al., 2004), these processes are under intensive investigation and may thus provide new opportunities for therapeutic intervention in the future.

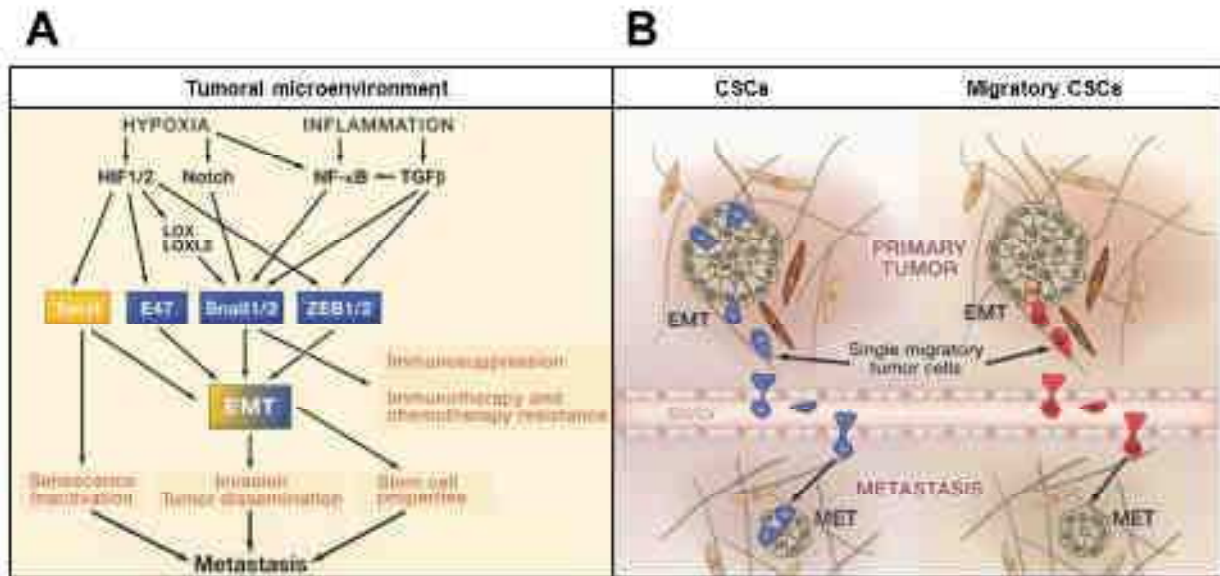


Figure 11: EMT and Tumor Progression

(A) EMT inducers promote metastasis formation. **(B)** EMT is now thought to play a fundamental role in tumor progression and metastasis formation. One challenge is to understand whether disseminating cells are cancer stem cells acting as tumor-initiating cells in the primary tumor (blue cells), if they are derived from somatic epithelial tumor cells that have undergone EMT to acquire stem cell-like properties (red cells), or some combination of these two possibilities. BV/LV, blood vessels/lymphatic vessels. Adapted from (Thiery et al., 2009).

In summary, a third of the CRC patients already present metastases at the moment of diagnosis, a stage at which this cancer is practically incurable. So although metastasis formation is the most important prognostic factor for patient survival, it is the least understood step in colorectal cancer. Invasion is the first step in the process leading to metastasis and is rendered possible by mutations but also by transient changes that are induced by the tumor micro-environment. This is a highly dynamic process that is very difficult to study as its important steps seem reversible. At the moment, we just have snap shots and new experimental approaches will be necessary to understand the plasticity of CRC cells and its impact on tumor progression.

III) Cdx2, a central player in intestinal development and homeostasis

Multiple signaling pathways are required to ensure the constant renewal of the gut and end up modulating the activity of numerous molecules, including transcription factors. One of those, encoded by the Cdx2 homeotic gene, is indispensable for the intestinal homeostasis.

1. Cdx2, the gene and the protein

1.1. The homeobox gene Cdx2

Homeobox genes encode transcription factors that play crucial roles in development by regulating patterns of anatomical structures. They have in common the homeobox, encoding the 60 aa-long homeodomain, arranged in 3 alpha-helices, that binds to the DNA. Despite the similar 3D structure, they present considerable variations in the homeodomain sequence (Abate-Shen, 2002).

Cdx1, Cdx2 and Cdx4 are the three members of the mammalian homeobox gene family related to the *Drosophila* gene *caudal* (Cdx= Caudal type homeobox). In humans, the Cdx2 gene is localized on the chromosome band 13q12 in the the ParaHox gene cluster, an ancient paralogue of the Hox cluster that is important for axial patterning during embryonic development. Only Cdx1 and Cdx2 are implicated in the development of the intestine and expressed in the adult colon and small intestine, even if Cdx4 is expressed transiently in the hindgut during embryogenesis (Guo et al., 2004; Rawat et al., 2008). Most studies focus on Cdx2 as it has been revealed that: 1) contrary to Cdx1, the loss of Cdx2 expression has a dramatic impact on embryonic development and intestinal homeostasis; 2) Cdx2 is implicated in cancers of the gastrointestinal tract and especially in colorectal cancer.

1.2. Protein structure of Cdx2

The Cdx2 gene is composed of 3 exons and encodes a 313 aa-long protein that can be divided in three main functional domains. The central region of Cdx2 is evolutionary conserved and corresponds to the homeodomain, which binds to DNA (Gautier-Stein et al., 2003). The N-terminal region acts as a transcriptional activator domain and modulates the transcriptional activity of Cdx2 (Rings et al., 2001; Taylor et al., 1997). The C-terminal region is less studied, but seems to be implicated in the regulation of Cdx2 activity (Calon et al., 2007b; Gross et al., 2008). The homeodomains of Cdx1 and Cdx2 are nearly identical (2-3 aa are different in human and murine Cdx1/2), whereas the percentage of identity is lower in the N-terminal (35.5%) and C-terminal (36.5%) domains flanking the homeodomain (Gautier-Stein et al., 2003; Calon et al., 2007b). Beside the full length protein, a splicing isoform of Cdx2 called miniCdx2 was recently described: this

isoform lacks the N-terminal transactivation domain, which is replaced by a different 13 aa extension (Modica et al., 2009; Renouf et al., 2012).

2. Expression and activity of Cdx2

The activity of Cdx2 depends on a combination of transcriptional and post-transcriptional regulations. In the last years, multiple factors regulating Cdx2 in the embryo and in the healthy adult intestinal epithelium were uncovered.

2.1. Cdx2 expression profile

Cdx2 expression is detected in mice as early as E3.5 in the extra-embryonic trophoctoderm. In the embryo itself, expression can be detected at E8.5 in several embryonic tissues: the posterior gut, the neural tube and the tail bud. Strikingly, from E12 on, Cdx2 expression is detected exclusively in the endoderm of the primitive gut where it is maintained through life. The level of Cdx2 expression peaks in the proximal colon and diminishes in either anteroposterior direction. Cdx2 expression is detected in intestinal stem cells (Stringer et al., 2012) and all the differentiated cell types of the intestinal epithelium, with the exception of Paneth cells which lack Cdx2 expression (Crissey et al., 2011). However, Cdx2 nuclear staining is stronger in the cells lining the villus than in the crypt in the small intestine; conversely, no such expression gradient could be observed in the colonic crypt. Cdx1 is expressed together with Cdx2 in the proliferative cells, but in contrast to Cdx2, its expression is decreased in the differentiating cells (Silberg et al., 2000).

2.2. Transcriptional regulation of Cdx2 expression

The complexity of Cdx2 transcriptional regulation was highlighted by *in vivo* promoter studies using transgenic mice. A 5kb promoter region was sufficient to activate Cdx2 expression from gastrulation to midgestation. However, from midgestation to the adult stage, a 9.3kb promoter fragment was required for intestine specific expression (Hinoi et al., 2003; Benahmed et al., 2008). Within this region, a 0.2kb sequence with binding sites for HNF4 α , GATA6, TCF4, and β -catenin was necessary for the activation of the Cdx2 promoter. The synergistic effect of these transcription factors on Cdx2 expression was demonstrated *in vitro* using reporter assays (Benahmed et al., 2008). Corroborating these data, it was later found that HNF4 α could bind to the Cdx2 promoter (Boyd et al., 2009). Another important regulator of Cdx2 expression is Cdx2 itself, as it has been shown that it could bind to its own promoter and activate transcription (Xu et al., 1999; Da Costa et al., 1999).

2.3. Extracellular factors regulating Cdx2 transcription

Interestingly, multiple extracellular factors were shown to modify Cdx2 expression *in vitro*. For

example, sodium butyrate, a byproduct of fiber fermentation by colonic bacteria (Domon-Dell et al., 2002), and the basement membrane protein Laminin 1, induced Cdx2 expression in colon cancer cell lines (Lorentz, 1997). Factors that are secreted by stromal myofibroblasts, like Wnt5a, also stimulated Cdx2 expression in intestinal cells (Pacheco and Macleod, 2008). However, some extracellular matrix proteins could also repress Cdx2 expression: for instance, Collagen I inhibited Cdx2 expression via a beta1 integrin/FAK signaling cascade (Brabletz et al., 2004). Taken together, these observations suggest that **Cdx2 expression *in vivo* might be dependent on the cellular micro-environment.**

2.4. Non-transcriptional regulation of Cdx2

2.4.1. Regulation of protein stability

Cdx2 levels are not only regulated on the transcriptional level, but also by post-transcriptional modifications affecting the protein's half-life. Indeed, phosphorylation of Cdx2 by kinases implicated in cell cycle progression, such as Erk1/2 and CDK2, leads to its polyubiquitinylation and degradation by the proteasome (Krueger et al., 2009). Conversely, in intestinal cells that start to differentiate, the cyclin-dependent kinase inhibitor p27^{Kip} stabilizes Cdx2 by preventing its phosphorylation by CDK2 (Boulanger et al., 2005; Gross et al., 2005). Of note, these data are consistent with the expression pattern of Cdx2 and its regulators in the intestine. Indeed, Cdx2 transcripts are uniformly detected by in situ hybridization along the crypto-villus axis, whereas Cdx2 and p27 proteins show an increased gradient of expression in differentiating cells, which correlates with the loss of CDK2 and ERK1/2 expression and probably activity.

2.4.2. Modification of the transcriptional activity

Not only Cdx2 protein levels but also its transcriptional activity can be modulated by post-translational modifications. For instance, the MAPK p38 α phosphorylates Cdx2 in differentiated cells and this leads to enhanced transcription of Cdx2 target genes (Houde et al., 2001). On the contrary, high levels of S60-phosphorylated Cdx2 are detected in the proliferative crypt cells and *in vitro* analysis showed that this phosphorylation actually inhibits Cdx2 transcriptional activity: this might explain why Cdx2 target genes are mainly activated in the upper third of the crypt, although no Cdx2 expression gradient is observed in colonic crypts (Rings et al., 2001; Silberg et al., 2000).

More recently, another way of regulating Cdx2 activity was revealed with the detection in the proliferative crypt cells of a dominant negative isoform of Cdx2 (miniCdx2), that lacks the transcription activator domain and whose fixation on the Cdx2 binding sites inhibits transcription by full-length Cdx2 (Modica et al., 2009; Renouf et al., 2012). Altogether, these examples show that

Cdx2 activity is regulated by a combination of diverse mechanisms and is not only reflected by mRNA or even protein levels.

3. Role of Cdx2 *in vivo*

3.1. Cdx2 Knockout mice

To study the function of Cdx2 *in vivo*, Chawengsaksophak et al made a first attempt by trying to generate Cdx2 Knockout mice. This was unsuccessful because **the homozygous gene invalidation in Cdx2^{-/-} mice turned out to be lethal** (Chawengsaksophak et al., 1997). Later it was shown that the absence of Cdx2 prevents trophectoderm maturation and consequently blastocyst implantation (Niwa et al., 2005). In contrast to homozygous Knockout mice, heterozygous Cdx2^{-/+} mice are viable, fertile and present no major dysfunctions. However, these mice presented **anterior homeotic shifts of their axial skeleton**, which means that a posterior element, such as a vertebra or a rib presents the morphology of a more anterior structure of the skeleton. In addition, these mice totally lost Cdx2 expression in some regions of the proximal colon and there developed lesions with intercalary growth. This resulted in the “filling in” of tissue types that exhibited properties of more anterior gastrointestinal tissues, such as esophageal and gastric tissues. Genomic analysis of these heteroplasia showed that there was no loss of heterozygosity, meaning that the reduction of Cdx2 expression was due to deregulation (Chawengsaksophak et al., 1997; Beck et al., 1999, 2003).

3.2. Cdx2 conditional Knockout mice

To circumvent the problem of embryonic lethality induced by total Cdx2 depletion, conditional Knockout mice for Cdx2 were created by several research groups, which permitted to study the consequences of Cdx2 loss at different stages of development and in the adult. All investigators used the CreLoxP system in which both Cdx2 alleles are flanked by loxP sites (Cdx2^{fl/fl}), allowing specific excision of Cdx2 in the tissues expressing the Cre recombinase. Because Cdx1 and Cdx2 can be functionally redundant, especially in the proliferating cells of the crypt, some authors made double Knockout mice for Cdx1 and Cdx2, using Cdx1^{-/-} mice. Cdx1^{-/-} mice are viable and show alterations of the skeleton, but have no intestinal phenotype (Savory et al., 2009b).

3.2.1. Consequences of Cdx2 loss during embryonic development

Savory and colleagues studied the role of Cdx2 during early embryonic development. They used Cdx2^{fl/fl} Cdx1^{-/-} mice that also expressed the tamoxifen-inducible CreERT2 in all tissues. Mice that were treated with tamoxifen at E5.5 died at E10 and had drastic development abnormalities. They presented abnormal axis elongation, neural tube closure defects and somite patterning alterations, demonstrating that Cdx2 is crucial for these events in early embryonic development (Savory et al.,

2011, 2009a, 2009b).

To study specifically the role of Cdx2 in the development of the gut, 3 groups used mice expressing the Cre recombinase in the intestinal epithelium (Table 2). Gao et al knocked out Cdx2 in the early endoderm so that Cdx2 could not be detected at E9.5. The most dramatic effect of Cdx2 loss was that the colon was not formed and the duodenum became dilated at E16.5 as a result of the intestinal obstruction. At E18.5, the number of villi was reduced in the duodenum and absent in the ileon and jejunum. The cell differentiation process was disturbed, which was illustrated by the absence of enterocytes, goblet cells or enteroendocrine cells, and loss of the brush border typical of absorptive intestinal cells. Instead of that, cells presented features of keratinocytes, the cells that constitute the esophageal epithelium (Gao et al., 2009).

Grainger et al induced the loss of Cdx2 expression later in development, at E13.5, using a tamoxifen-inducible Cre under the control of the intestine-specific Villin promoter. At this stage, colon formation could occur, but there were still dramatic consequences on the intestinal development. Observation at E18.5 showed that the duodenum was distended and histological analysis of the small intestine indicated that the epithelium of mutant mice was disorganized and villi were shortened. In the duodenum, the H^+/K^+ -ATPase, characteristic of stomach epithelium, was detected on the apical side of villi, gastric genes like Sox2, Muc1, Grehlin1 were up-regulated, whereas intestinal markers were down-regulated (Grainger et al., 2010). These observations indicate that the severity and the degree of transdifferentiation decrease when Cdx2 is lost at a later stage of development.

This was further supported by a study in which Cdx2 was ablated at E15.5 in the intestinal epithelium. This resulted in a reduction of the villi length and analysis of the ultrastructure of cells revealed that microvilli of enterocytes were shortened, less abundant and disorganized. In particular, the Actin cytoskeleton core of the microvilli was altered, showing reduced Actin bundling. Polarization was also altered as alkaline phosphatase activity, normally found only on the apical side of enterocytes, was detected on the basolateral side. However, in contrast to Cdx2 invalidation at earlier embryonic stages, no gastric or esophageal transdifferentiation occurred (Gao and Kaestner, 2010).

Nevertheless, all these conditional Knockout mice did not survive longer than P2 and presented severe abnormalities in the development and function of the intestine, that were more dramatic

when Cdx2 expression was lost earlier. Altogether these results lead to the conclusion, **that Cdx2 imposes intestinal identity between E9.5 and E15.5** because the loss of Cdx2 in the early endoderm or at E13.5 allows an ectopic foregut differentiation program with esophageal or stomach-like differentiation. This is in line with the observation that the lesions that are found in the proximal colon of Cdx2^{-/+} mice (filling in of anterior tissue types in the colon) are already present at birth and no new lesions develop. Later, Cdx2 is important for intestinal epithelial differentiation and function but its loss can no more induce a total conversion to a more anterior cell fate.

3.2.2. Consequences of Cdx2 loss in the adult

To better define its role in intestinal tissue homeostasis in the adult, different research groups conditionally inactivated Cdx2 in mice using tamoxifen-responsive Cre under the control of promoters specific of the intestinal epithelium: this tissue specific Cre recombinase was activated by tamoxifen injections in the adult (Table 3).

Upon efficient Knockout of Cdx2 in the adult intestine, mice had chronic diarrhea and died of starvation at the latest 3 weeks after the start of tamoxifen treatment. The weight loss was caused by malabsorption, indicating that loss of Cdx2 impairs the function of enterocytes. Intestinal villi of Knockout mice were shortened and the microvilli on absorptive cells were shorter and less dense than in their wild-type littermates (Hryniuk et al., 2012; Verzi et al., 2011). Another consequence of Cdx2 loss was the reduction of the number of proliferating cells in the crypt (Verzi et al., 2011; Hryniuk et al., 2012; Stringer et al., 2012). Although transdifferentiation into stomach-like tissue was not observed, analysis of the gene expression profiles of Cdx2^{-/-} mice demonstrated that stomach-specific gene expression was up-regulated compared to wild-type littermates (Verzi et al., 2011; Stringer et al., 2012).

As most parts of their intestinal epithelium were not affected, mice with only partial Cdx2 Knockout did not die of starvation, allowing long-term studies. Indeed, these mice exhibited some zones of the epithelium that lacked Cdx2 expression and presented an abnormal crypt-villus axis with aberrant cell morphology. Moreover, specific markers of the gastric epithelium (H⁺/K⁺-ATPase, Pepsidogene C, Claudin-18), were detected by immunohistochemistry (Hryniuk et al., 2012; Stringer et al., 2012). These results demonstrate that the intestinal epithelium retains some plasticity to acquire stomach-like properties, but a total conversion to a gastric epithelium, as observed after the loss of Cdx2 expression during the early development of the intestinal epithelium, is not possible in the adult. Importantly, these studies also show that **Cdx2 activity on proliferation and differentiation is required throughout life to ensure the functionality of the intestinal epithelium.**

3.3. Cdx2 transgenic mice

3.3.1. Overexpression in the intestinal epithelium

Overexpression of Cdx2 has also deleterious effects on intestinal tissue homeostasis. Transgenic Villin-Cdx2 mice, overexpressing Cdx2 specifically in the intestinal epithelium, failed to develop Paneth cells, had an increased number of goblet cells, presented alterations in the organization of the crypt base and had problems to metabolize triglycerides. These mice also exhibited postnatal weight gain delay and had an increased risk of death before weaning (Crissey et al., 2011).

3.3.2. Overexpression in non-intestinal tissues

Since Cdx2 is ectopically expressed in certain diseases of the stomach or esophagus (see below, page 52), transgenic mice expressing Cdx2 specifically in these tissues were created. Ectopic expression of Cdx2 in the stomach of mice induced the transdifferentiation of the gastric epithelial cells into enterocyte-like cells (Mutoh et al., 2002). Similarly, ectopic expression in the squamous epithelium generated transitional cells resembling the typical columnar Barrett's esophagus cells (Kong et al., 2011). These findings indicate that **gain of Cdx2 expression can induce an intestinal cell fate** in cells of the gastrointestinal tract and that these tissues maintain a certain degree of plasticity.

In summary, the analysis of various animal models showed that Cdx2 is required for the implantation of the embryo, axial patterning and elongation, as well as organogenesis of the intestinal tract. It became also evident that at all stages, embryonic and adult, complete loss of Cdx2 is lethal. In the embryo, absence of Cdx2 expression in the developing intestinal epithelium prevents its differentiation and allows the acquisition of features typical of more anterior tissues (esophagus, stomach). In the adult, depletion of Cdx2 in the intestinal epithelium inhibits correct cell differentiation, leading to death by starvation. Cdx2 is required to maintain a functional intestinal epithelium, by controlling features like villus length, cell localization, ultrastructure of enterocytes, intercellular adhesion, apico/basal polarization and production of digestive enzymes. In addition, loss of Cdx2 expression can lead to the expression of stomach specific genes in the intestinal epithelium, whereas ectopic expression of Cdx2 in anterior tissues like stomach and esophagus can induce intestinal features. Taken together, these observations establish Cdx2 as one of the master regulators of intestinal identity and differentiation.

| Ref. | Targeted Tissue | Cre-promoter | Cdx2 loss | Phenotype | Observed in all studies |
|------------------|-----------------------|--------------------|-----------|--|---|
| Gao 2009 | Early endoderm | Foxa3 constitutive | E 9.5* | Death at P0 No colon formation Dilated duodenum Increased number of cycling cells Cycling cells in the whole villus Esophageal features | No or shortened villi Decrease in microvilli density Defects in apico-basal polarization |
| Grainger 2008 | Intestinal epithelium | Villin inducible | E 13.5 | Death at P0 Dilated duodenum Cycling cells in the whole villus Stomach-like identity | |
| Gao 2010 | Intestinal epithelium | Villin inducible | E 15.5 | Death at P1 or P2 No Actin bundling in microvilli Perturbation of lysosomal transport | |

Table 2: Conditional Cdx2 Knockout mice – Embryonic Development

Mice with loxP sites flanking both alleles of Cdx2 (Cdx2^{fl/fl}) were crossed with mice expressing Cre (Gao 2009) or inducible Cre-ER^{T2} (Grainger, Verzi, Gao 2010).

E –embryonic day P- postnatal day

* The Cre in this construction is not inducible. Foxa3 is expressed in the early endoderm before Cdx2. In consequence, the Cdx2 exon is excised by the Cre before Cdx2 starts to be expressed at E 9.5 in the primitive gut.

| Ref. | Targeted Tissue / Cell type | Cre-ER ^{T2} promoter | Cdx KO | Survival after KO | Phenotype |
|--------------------|-----------------------------|-------------------------------|---|---------------------|---|
| Verzi 2010/2011 | Intestinal epithelium | Villin | Cdx2 ^{-/-} Cdx1 ^{+/+} | 3 weeks | Mice die of starvation Altered structure and decreased number of microvilli Shortened villi |
| | All intestinal cells | Normal induction | Cdx2 ^{-/-} Cdx1 ^{-/-} | 1 week | |
| Hryniuk 2011 | Intestinal epithelium | Villin | Cdx2 ^{-/-} Cdx1 ^{+/+} | 5-6 days | Decreased proliferation in the crypt ** Distension of the intestine Defects in polarization |
| | | Normal induction | Cdx2 ^{-/-} Cdx1 ^{-/-} | 5-6 days | |
| | All intestinal cells | Villin Weak induction | Cdx2 ^{-/-} Cdx1 ^{-/-} | Not lethal | After 6 weeks appear zones devoid of Cdx2 expression, without mature enterocytes and presenting stomach-like features |
| Stringer 2012 | Intestinal epithelium | AH | Cdx2 ^{-/-} Cdx1 ^{+/+} | Not lethal | Only 35% (Lgr5) or 70% (AH) of cells are Cdx2 negative Gastric genes are expressed Reduced cell proliferation |
| | All intestinal cells* | Normal induction | | Mice remain healthy | |
| | Intestinal epithelium | Lgr5 | Cdx2 ^{-/-} Cdx1 ^{+/+} | Not lethal | No gastric differentiation Cyst formation in the intestinal epithelium |
| | Intestinal stem cells | Normal induction | | Mice remain healthy | |

Table 3: Conditional Cdx2 Knockout mice

Adult Mice with loxP sites flanking both alleles of Cdx2 (Cdx2^{fl/fl}) were crossed with mice expressing inducible Cre-ERT2.

* Except Paneth cells ** In the Cdx2^{-/-} Cdx1^{+/+} mice by Verzi reduced proliferation was not observed

4. Mode of action of Cdx2 in intestinal cells

4.1. Cellular function

4.1.1. Cdx2 promotes differentiation

Epithelial cell differentiation is indispensable for proper intestinal function. Hallmarks of differentiation of intestinal cells are: apico/basal polarity, cell type specific morphology (Ex: microvilli), expression of intestinal enzymes, hormones, mucus proteins or transporters and strong intercellular adhesion that ensures barrier function of the intestinal epithelium.

As described above (see III)3., page 36), *in vivo* modification of Cdx2 expression has spectacular consequences on intestinal cell differentiation that actually correlate with numerous observations made on intestinal cells lines cultivated *in vitro*. The most impressive demonstrations of the differentiating potential of Cdx2 were made in the undifferentiated colon cell lines IEC-6 and COLO 205, in which forced Cdx2 expression induced drastic morphological changes: cells acquired a polarized, columnar shape with apical microvilli covered by a glycocalyx, produced various digestive enzymes and formed tight, adherens and desmosomal junctions.

More recently, Gao and colleagues specifically demonstrated the effect of Cdx2 on apico/basal polarity using a 3D culture system. Indeed, silencing of Cdx2 by shRNA cells in Caco-2 cells grown in matrigel led to the formation of an epithelial ball instead of a cyst-like structure organized around a fluid-filled central lumen: observation of various basolateral markers (Ex: E-cadherin, β -catenin, Na^+/K^+ -ATPase) and of the apical Par polarity complex indicated impaired apical–basal polarity upon loss of Cdx2, which was associated with defective apical transport. Accordingly, Cdx2 depletion in conditional Knockout mice, led to the formation of large cytoplasmic vacuoles in intestinal cells and down-regulation of genes involved in endo-lysosomal function (Gao and Kaestner, 2010; Verzi et al., 2011).

4.1.2. Cdx2 inhibits other cellular processes such as proliferation and migration

A multitude of *in vitro* studies demonstrated that Cdx2 expression reduced anchorage-dependent or anchorage-independent growth of normal, adenoma and carcinoma epithelial cells (Mallo et al., 1998; Hinoi et al., 2003; Gross et al., 2005, 2008). Further analysis showed that Cdx2 could block the G0/G1-S progression in intestinal cell lines (Aoki et al., 2003, 2011), implying an impact on cell proliferation. However, a pro-apoptotic effect of Cdx2 was also observed in various intestinal contexts (Mallo et al., 1998; Bonhomme et al., 2003; Renouf et al., 2012) and thus may also

contribute to a reduced cell number. Of note, the activity of Cdx2 on cell growth appears to be dependent on the context and cell type: for instance, somatic Knockout of Cdx2 reduced anchorage-independent growth of LOVO intestinal cells (Dang et al., 2006) and shRNA silencing of Cdx2 expression inhibited the proliferation of various human leukemia cell lines (Scholl et al., 2007).

Cdx2 was also shown to inhibit intestinal cell migration and invasion using Boyden Chambers coated or not with Matrigel (Gross et al., 2008; Ezaki et al., 2007). Unexpectedly, Cdx2 expression was proposed to influence chromosome segregation, as well as NHEJ (Non Homologous End Joining) DNA damage repair in intestinal cells (Aoki et al., 2011; Renouf et al., 2012).

Taken together, the results obtained *in vitro* are generally consistent with the biological functions of Cdx2, inferred from expression pattern and animal models. The impact on proliferation and migration may however be questioned as some expression of Cdx2 is detected in proliferative cells of the crypt and as differentiated cells seem to migrate up the villus. However, Cdx2 expression is sharply decreased during *in vitro* wound healing of colon cancer Caco-2/TC7 cells, a process that relies on enhanced migration and proliferation (Gross et al., 2008). It is also important to realize that the biological activity of Cdx2 does not just correspond to the sum of the “cellular activities” of Cdx2 observed *in vitro* which might sometimes correspond only to secondary consequences (Ex: increased adhesion might explain reduced migration) and depend on the cellular context.

4.2. Molecular function of Cdx2

4.2.1. Direct transcriptional targets of Cdx2

The main function of Cdx2 is to activate gene expression in the embryo and later in the intestinal epithelium. The consensus binding site of Cdx2 is (C/TATAAAG/T), an AT-rich sequence typical of homeobox proteins, in direct or reverse orientation (Margalit et al., 1993). However, Cdx2 can also bind to sequences that are slightly different (Alkhoury et al., 2005; Verzi et al., 2010b). In the last years, many direct Cdx2 target genes were identified, meaning that Cdx2 binds directly to its site on the promoter region of these genes, at least in intestinal cell lines.

During early development, Cdx2 regulates antero-posterior patterning by stimulating the expression of various Hox genes such as Hoxa5 (Tabariès et al., 2005). Later, in the developing or mature intestinal epithelium, Cdx2 regulates a large number of genes involved in intestinal identity and in various intestinal functions (16 0877 genes identified in Caco2 cells according to Verzi (Verzi et al., 2010b)). Indeed, Cdx2 regulates the transcription of genes implicated in cell fate decision, such as

the Notch ligand Dll1 (Grainger et al., 2012), the transcription factors Math1 (Mutoh et al., 2006) or KLF4 (Dang et al., 2001; Modica et al., 2009) and even itself (Xu et al., 1999; Da Costa et al., 1999).

Since Cdx2 is critical for enterocyte maturation, the first direct target genes identified encoded digestive enzymes like Sucrase-isomaltase (Suh et al., 1994), Lactase (Fang et al., 2000) or PhospholipaseA/lysophospholipase (Taylor et al., 1997). Many transporters, necessary for the absorption and secretion of nutrients by enterocytes, are also Cdx2 target genes: for instance the iron transporter Hephaestin (Hinoi et al., 2005), the Multidrug Resistance 1 protein (MDR1/P-glycoprotein/ABCB1) (Takakura et al., 2010) or the Solute carrier family 5, member 8 (SLC5A8), (Kakizaki et al., 2010).

Other Cdx2 target genes are involved in the modeling of the typical mucus-covered brush border: they encode for example the actin-binding protein Villin (important for microvilli architecture (Yamamichi et al., 2009)) and mucus constituents such as Muc2 (Yamamoto et al., 2003) and Muc4 (Jonckheere et al., 2007). Some Cdx2 target genes encode adhesion molecules, potentially involved in intestinal barrier function and cell polarization: several members of the Cadherin superfamily such as LI-cadherin (Hinoi et al., 2002) and Desmocollin 2 (Funakoshi et al., 2008), but also Claudin-2 (Sakaguchi et al., 2002) and Claudin-1 (Bhat et al., 2012). Finally, the transcription of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 can be stimulated by Cdx2 and thus may contribute to the anti-proliferative effect of Cdx2 (Bai et al., 2003).

4.2.2. Cdx2 interacts with other transcription factors to regulate gene expression

The activation of Cdx2 target genes exhibits spatiotemporal variations, depending on the different compartments of the intestinal epithelium and the different life phases (before birth, after birth and after weaning) considered. This is in part due to the fact that the activity of Cdx2 is fine-tuned by its interaction with other transcription partners. For example, the Sucrase-isomaltase expression increases dramatically after the weaning period and only in the small intestine, but Cdx2 expression does not differ in the same way. Boudreau et al showed that the Sucrase-isomaltase gene is regulated by a combination of Cdx2, expressed in all intestinal cells, GATA4, detected in the villus of the small intestine but not in the colon, and HNF1 α , whose expression starts after weaning. Interestingly, to regulate intestine-specific gene expression, members of the GATA and HNF family often collaborate and directly interact with Cdx2 (Ex: Claudin-2, Muc4) (Boudreau et al., 2002; Sakaguchi et al., 2002; Jonckheere et al., 2007).

Similarly, in a recent study by Verzi et al, the different Cdx2 partners and target genes were systematically analyzed in the crypt and villus of the small intestine. In the proliferating cells, Cdx2 co-occupied regions together with GATA6 and primarily activated genes implicated in development,

whereas in the differentiated cells, Cdx2 was associated with HNF4 α and HNF1, and mainly activated genes implicated in metabolism. Accordingly, the partners and the sites occupied by Cdx2 in the genome of differentiated and proliferating intestinal Caco2 cells differed substantially and the number of genes regulated by Cdx2 was by far more important in differentiated cells than in proliferative cells (Verzi et al., 2010b).

Beside GATA and HNF transcription factors, Cdx2 also interacts physically and/or functionally with other partners, like for example Cdx1, which regulates, together with Cdx2, expression of the transporter SLC5A8 (Kakizaki et al., 2010) and of Desmocollin 2 (Funakoshi et al., 2008). Recently, the results of a genome-wide CHIP-chip analysis in colonic cells suggested that Cdx2 and TCF4 (the principal effector of the Wnt/ β -catenin signaling in the intestine) commonly interact with the same cis-regulatory regions to activate intestine specific transcription (Verzi et al., 2010a).

Of note, Cdx2 generally stimulates gene expression, but can also in rare cases repress transcription. For example Cdx2 can inhibit expression of Intestinal Alkaline phosphatase (Alkhoury et al., 2005) or Glucose-6-phosphatase (Calon et al., 2007b; Gautier-Stein et al., 2003) induced by Cdx1. By binding to the Cdx consensus site, without inducing gene expression, Cdx2 acts in a dominant negative way.

4.2.3. Molecular functions of Cdx2 independent of DNA binding

Cdx2 does not necessarily bind to the DNA to activate gene transcription. For example, SP1 and Cdx2 have a synergistic effect on the activation of Pept 1 expression, an H⁺/peptide co-transporter located on the brush border of the small intestine. However, Cdx2 does not directly bind to the promoter but to the transcription factor SP1, which binds on its consensus site on the Pept 1 promoter (Shimakura et al., 2006).

Moreover, new findings extended the known function of Cdx2, as they showed that it could act on transcription without using its transcriptional activator properties. For instance, Cdx2 affects the Wnt signaling pathway by direct interaction with β -catenin, thereby inhibiting the formation of the β -catenin/TCF4 complex and consequently Wnt target gene activation (Guo et al., 2010). Another example of transcriptional repression by Cdx2 independent of DNA binding has been reported by Kim et al. In their study, Cdx2 inhibited COX-2 expression by interacting with the p65 sub-unit of NF- κ B, which prevented the binding of NF- κ B on the COX-2 promoter and its transcription (Kim et al., 2004; Mutoh et al., 2007).

4.2.4. Molecular functions of Cdx2 independent of transcription

In the preceding examples, Cdx2 is still acting indirectly on gene transcription. However, in the last years, several studies revealed novel and unexpected activities of Cdx2, which go beyond its role as an intestine specific transcription factor. Recently, Renouf et al reported that Ku70/Ku80, a protein complex important for the initiation of DNA repair by the non-homologous DNA end joining (NHEJ) process, interacted with Cdx2 in colon cancer cells via its homeodomain. Cdx2 did not affect the recruitment of Ku proteins, but nevertheless inhibited the activity of the DNA repair complex (Renouf et al., 2012).

Cdx2 can also affect proteins without direct interaction. This was discovered by Aoki et al who generated Cdx2 mutants by inserting point mutations in the homeodomain. These mutated Cdx2 proteins localized in the cell nucleus, but were unable to bind DNA and activate transcription. Strikingly, these mutants still inhibited cell proliferation, actually to the same extent than wild-type Cdx2. Further investigations revealed that wild-type Cdx2 and the mutant proteins stabilized the cell cycle inhibitor p27^{KIP} by inhibiting its polyubiquitylation and sub-sequent proteolysis by the proteasome (Aoki et al., 2011).

4.2.5. Unresolved mechanisms by which Cdx2 affects the cellular phenotype

In some cases, the exact mechanism of Cdx2 activity is not understood but might be important for intestinal homeostasis. One example is the potential repression of the mTOR pathway by Cdx2: Aoki et al proposed that the loss of Cdx2 stimulated the activity of this pathway, critical for cell proliferation and survival, thereby accelerating cell cycle progression and chromosomal segregation defects, but they could not decipher how exactly Cdx2 acted (Aoki et al., 2003). Another example is the enhanced trafficking of E-cadherin to the membrane of colon cancer cells, which strengthens Ca²⁺-dependent adhesion: the exact mechanism has not been elucidated but it has been shown that Cdx2 reduced the phosphorylation of β -catenin and p120 catenin, thereby modulating their binding to E-cadherin (Ezaki et al., 2007).

In summary, many known target genes of Cdx2 are important factors for intestinal cell differentiation and polarization, explaining quite clearly how Cdx2 determines the identity of the intestinal epithelium and regulates enterocyte function. According to recent studies, Cdx2 acts not only as a transcription factor. Yet, how Cdx2 inhibits cell proliferation and migration at the molecular level remains a puzzling question. Actually, the molecular consequences of the absence of Cdx2 in physiopathological conditions such as colon cancer are far from being understood.

5. Cdx2 and colorectal cancer (CRC)

5.1. Cdx2 expression in CRC

Genes implicated in embryonic development, like the homeobox genes, are often implicated in tumorigenesis and cancer progression. In general, homeobox genes that are expressed in undifferentiated cells are up-regulated in tumors, and genes expressed in differentiated cells, down-regulated (Abate-Shen, 2002). As a homeotic transcription factor important for intestinal development and for the regulation of the proliferation/differentiation balance in the adult intestinal epithelium, Cdx2 was suggested to be involved in colorectal cancer.

5.1.1. Cdx2 expression in primary tumors

In immunohistochemical analyses, Cdx2 is revealed as a strong nuclear staining in cells of the healthy small intestine and colon. In nearly all colorectal adenocarcinomas a certain level of Cdx2 expression can be detected, but this expression is very heterogeneous, from one tumor to another. In addition, within the same tumor, some cells exhibit strong nuclear Cdx2 staining, in others it is weaker or even lost. In many cells, Cdx2 expression persists but becomes diffuse and cytoplasmic (Hinoi et al., 2001; Lugli et al., 2008; Kaimaktchiev et al., 2004; Choi et al., 2006; Bakaris et al., 2008; Brabletz et al., 2004; Knösel et al., 2012; Ee et al., 1995). This heterogeneous expression of Cdx2 explains why conflicting results have been obtained in different studies. Indeed, the data were highly dependent on the set of tumors considered and the criteria used to evaluate the tumors as positive or negative for Cdx2 expression. Anyhow, reduced expression of Cdx2 could be associated with high microsatellite instability status (MSI) (Hinoi et al., 2001; Lugli et al., 2008), advanced tumor stage, higher tumor grade (Ee et al., 1995; Kaimaktchiev et al., 2004), lymph node metastasis (Choi et al., 2006; Bakaris et al., 2008) and reduced survival (Baba et al., 2009; Knösel et al., 2012).

Of note, down-regulation of Cdx2 expression was also clearly observed in tumors of nude mice arisen from xenografted colon cancer cells, chemically-induced colon carcinoma of rats or mice and spontaneous intestinal tumors of $Apc^{\Delta 716}$ mice (Brabletz et al., 2004; Ee et al., 1995; Bonhomme et al., 2003; Aoki et al., 2003).

5.1.2. Cdx2 expression in invasive cells and metastasis

Several studies reported that Cdx2 expression is decreased more systematically in cells located at the tumor front or disseminated in the adjacent stroma compared to the cells of the tumor center (Brabletz et al., 2004; Laurent et al., 2011; Karamitopoulou et al., 2011). Strikingly, most of the time, metastases (lymph nodes, liver) exhibited a similar level of Cdx2 expression than the primary tumor

(Brabletz et al., 2004) (**Figure 12**). Although, no lineage tracing experiment has been yet performed to prove that the identified invasive cells are the ones forming the metastases, **this suggests a dynamic expression pattern of Cdx2 during tumor progression, with a specific but transient reduction in invasive cells**. Of note, parallel changes in the expression or localization of several molecules (Ex: nuclear localization of β -catenin, cadherin switch) were observed in cells of the tumor invasive front and colon cancer cells involved in wound healing *in vitro*: Cdx2 expression was also similarly down-regulated in both contexts (Gross et al., 2008).

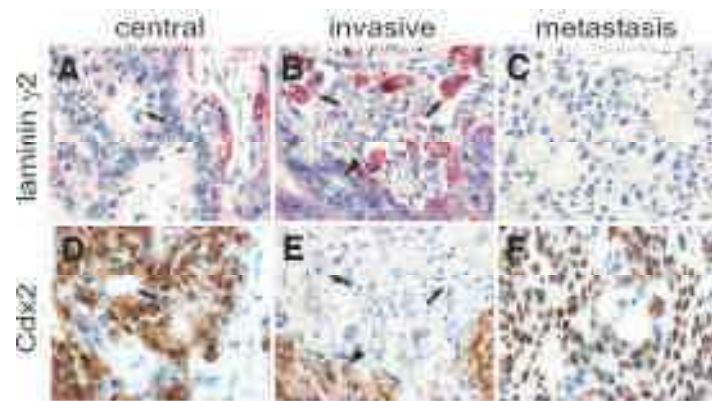


Figure 12: Lamininy2 chain and Cdx2 expression are transiently down-regulated in invasive cells

Immunohistochemical analysis for laminin γ 2 chain and Cdx2 was realized on serial sections of the center of a colon tumor. Shown are sections from the tumor center (A, D), the invasive front (B, E) and a liver metastasis (C, E) of the same patient. Arrows (AD): differentiated glandular structures, arrows (BE): invasive cells, arrowhead (BE): tumor center (Brabletz et al. 2004).

5.2. Mechanism of Cdx2 loss of expression in colon cancer

5.2.1. Type of mechanism involved in Cdx2 loss of expression in colon cancer cells

Deletions or mutations at the Cdx2 locus occur very rarely in colon tumors (Da Costa et al., 1999; Yagi et al., 1999; Wicking et al., 1998; Rozek et al., 2005; Sivagnanasundaram et al., 2001). Surprisingly, it has even been shown that in most CIN tumors there was a gain of Cdx2 copy number, but this gene amplification did not correlate with Cdx2 expression (Subtil et al., 2007). On the other hand, Hinoi and colleagues used somatic cell hybrids to show that silencing of Cdx2 expression was transferable upon cell fusion, **suggesting a dominant repression mechanism** (Hinoi et al., 2003). Since no epigenetic modifications of the Cdx2 promoter have been detected in colon cancer cell lines (Subtil et al., 2007; Hinoi et al., 2003), the existence of a transcriptional repression pathway is likely. Of note, such a regulatory mechanism would be consistent with a transient change of Cdx2 expression in invasive cells. Nevertheless, the mechanism by which Cdx2 expression may be reduced in the invasive colon cancer cells and reexpressed in metastasis remains elusive and requires further investigations.

5.2.2. Oncogenic pathways can down-regulate Cdx2 expression in colon cancer cells

Differential regulation of Cdx2 expression may result from alterations of signaling pathways in tumor cells. Several well-known oncogenic signaling pathways were indeed shown to repress Cdx2 expression in human colon cancer cell lines. For example, the PI3K signaling pathway, which is often constitutively activated in CRC, decreased Cdx2 expression, by favoring a p65/p50 NF- κ B heterodimer, which inhibited Cdx2 transcription (Kim et al., 2002). The Raf-Mek-ERK1/2 pathway, which is also hyper-activated in a large fraction of colon tumors due to mutations in K-Ras, also down-regulated Cdx2 expression through activation of PKC and AP1 binding sites on the Cdx2 promoter (Krueger et al., 2009; Lorentz et al., 1999). SOX9, whose expression is stimulated by the Wnt pathway and that is deregulated in nearly all colon tumors, indirectly inhibited Cdx2 transcription in intestinal cells by an unknown mechanism (Blache et al., 2004). Finally, given that disseminating colon cancer cells have most likely gone through EMT, transcriptional repressors inducing EMT may be involved in the systematic decrease of Cdx2 expression in invasive cells: indeed Slug, Snail and Zeb1 could repress Cdx2 transcription *in vitro* (Gross et al., 2008; personal communication). Taken together, these data confirmed that **aberrant activation of several oncogenic pathways in colon cancer cells is able to decrease the transcription of Cdx2**. However, no study has been yet performed to show a correlation in human colon tumors.

5.2.3. The micro-environment affects Cdx2 expression

The micro-environment is one of the driving forces of tumor progression in colorectal cancer. Immune cells producing cytokines, mesenchymal cells secreting growth factors, the extracellular-matrix, inflammation or even hypoxia can deregulate gene expression and consequently the behavior of cancer cells (Wels et al., 2008; Barker and Clevers, 2001).

In vitro, several micro-environmental factors linked to tumor progression were shown to modify Cdx2 transcription. For example, Collagen I, which is present in the lamina propria and is normally not in direct contact with the intestinal epithelium, can inhibit Cdx2 expression via a β 1 integrin/FAK pathway and thus might be, following the loss of the basement membrane, involved in the down-regulation of Cdx2 in disseminating cells (Brabletz et al., 2004). In addition, recent results obtained in our laboratory indicate that hypoxia can down-regulate Cdx2 transcription independently of HIF-1 α in colon cancer cell lines (Derbal et al., in revision).

The influence of the tumor environment on Cdx2 expression was further confirmed *in vivo* by grafting colon cancer cell lines and primary colon tumors under the skin or in the cecum wall of nude mice. Cells expressing Cdx2 when grown *in vitro* (T84 cells) lost Cdx2 expression when they were

injected into the cecum wall. Inversely, cells without Cdx2 expression *in vitro* (HT29 cells) gained Cdx2 expression after injection under the skin or in the liver of nude mice. In addition, *in vitro* co-culture experiments of colon cancer cells with skin fibroblasts increased Cdx2 expression, in contrast to duodenal fibroblasts, which down-regulated Cdx2 (Benahmed et al., 2007), corroborating the influence of the cecum/sub-cutaneous micro-environment. The involved factors were not identified, but the changes in Cdx2 expression were not due to clonal selection because the cells in these experiments were genetically stable and because the gain/loss of expression was reversible: cells that gained Cdx2 expression under the skin of mice, lost Cdx2 expression again after dissection of the tumor and injection of the resulting cells into the cecum wall or *in vitro* culture (Benahmed et al., 2007). This set of experiments elegantly shows **the importance of the tumoral micro-environment for Cdx2 expression and, importantly, the plasticity of Cdx2 expression in colon cancer cells.**

5.3. Cdx2 acts as a tumor suppressor in the colon

As Cdx2 expression is down-regulated in colon tumors and impacts on cell proliferation and migration, it was hypothesized that Cdx2 could act as a tumor suppressor in the colon. However, the “intestinal polyps” developed by Cdx2^{+/-} mice turned out to be non-neoplastic heteroplasias. This suggests that the loss of Cdx2 alone is not sufficient to initiate tumor formation, but it should be kept in mind that only one allele was invalidated in these mice. So far, none of the conditional homozygous Knockout mice were reported to form tumors, even if the tamoxifen-inducible CRE might not be ideal to observe such a long-term phenotype. To test if Cdx2 could influence tumor progression, Aoki et al crossed Cdx2^{+/-} mice with APC^{+/ Δ 716} mice. APC is an important tumor suppressor gene in the intestine and APC^{+/ Δ 716} mice develop adenomatous polyps, but mostly in the small intestine. Strikingly, Cdx2^{+/-}/APC^{+/ Δ 716} mice formed six times more adenomatous polyps, which were located in the distal colon (Aoki et al., 2003). The tumor suppressor activity of Cdx2 was also demonstrated in a different animal model by Bonhomme et al who treated Cdx2^{+/-} mice with azoxymethane (AOM), a chemical agent inducing colon carcinomas. The heteroplasias that Cdx2^{+/-} mice spontaneously developed in the proximal colon presented no modifications after treatment with AOM, providing additional evidence that they were not pre-cancerous lesions. However, 12 weeks after AOM treatment, Cdx2^{+/-} mice, but not their wild-type littermates, developed numerous malignant tumors in the distal colon (Bonhomme et al., 2003).

Importantly, injections of colon cancer cells expressing or not Cdx2 in nude mice not only showed the tumor suppressor activity of Cdx2, but also its anti-metastatic effect: indeed, forced expression of Cdx2 in colon cancer cells correlated with increased numbers of metastasis-free mice (Gross et al., 2008).

Obviously, Cdx2 cannot be considered as a classic tumor suppressor gene, since its locus is rarely deleted or mutated in colon tumors and the loss of its expression does not induce spontaneous tumors. **Nevertheless, experimental evidences indicate that Cdx2 expression has important consequences for tumor (speed, number, location) and metastasis formation.**

5.4. Mechanism of tumor suppression by Cdx2 in the colon

Analysis of the tumors formed in mice having an inactivated Cdx2 allele gave first hints concerning the mechanisms involved in the suppression of tumorigenesis by Cdx2. Indeed, the Cdx2^{+/-} mice treated with AOM had decreased apoptosis levels after irradiation compared to wild-type mice (Bonhomme et al., 2003), which is consistent with the findings of Renouf et al. that Cdx2 inhibits DNA repair and cell survival after cytotoxic stress *in vitro* (Renouf et al., 2012). Similarly, reduced apoptosis was observed in polyps of APC^{+/ Δ 716} Cdx2^{-/+} mice compared to single Knockout or wild-type mice. In addition, the reduction of Cdx2 in these compound mice stimulated proliferation and activation of the mTOR pathway, increasing chromosomal instability (see III)4.2.5, page 46) (Aoki et al., 2003). The increased proliferation may be linked to the enhanced activity of the mTOR pathway but also to the regulation by Cdx2 of the transcription or stability of several cyclin dependent kinase (CDK) inhibitors, such as p21/WAF1/CIP1 (Bai et al., 2003) or p27^{Kip} (Aoki et al., 2011). Alternatively, Cdx2 might reduce proliferation by blocking the transcriptional activity of β -catenin on genes associated with cell cycling such as Cyclin D1 or c-Myc (Guo et al., 2010).

Concerning the inhibition of metastasis by forced expression of Cdx2 (Gross et al., 2008), it was proposed that reduction of motility and invasion capacity of colon cancer cells observed *in vitro* were involved. Decreased motility might result from enhanced cell adhesion through the regulation of several adhesion molecules by Cdx2 (Claudin-2, LI-cadherin, Dcs-2, E-cadherin). Decreased invasion might be linked to enhanced metalloproteases production by colon cancer cells (MMP-2, MMP-7) in absence of Cdx2 as observed *in vitro* (Gross et al., 2008; Zheng et al., 2011; Brabletz et al., 1999).

Thus, despite the existence of several mechanisms potentially responsible for the anti-oncogenic and/or anti-metastatic effect of Cdx2, further investigation will be required to demonstrate which of these mechanisms are critical *in vivo*, especially in human colon tumors.

*In summary, Cdx2 expression is generally reduced in a heterogeneous manner in colon tumors, but this decrease becomes more systematic in invasive cells at the tumor front. The mechanism of Cdx2 down-regulation in tumor cells is not elucidated but might be driven by transcriptional repression. The inactivation of one allele of Cdx2 does not by itself initiate spontaneous tumor formation. However, reduced Cdx2 expression clearly accelerates or increases tumor formation in mice models of colon cancer. In addition, forced expression of Cdx2 was able to reduce the incidence of metastasis in nude mice. Cdx2 might oppose tumor progression by one or several of its reported activities (pro-apoptotic, pro-differentiating, anti-proliferative and anti-migratory), but **the processes involved in vivo are still unclear**. In regard of the deleterious effect of metastasis in colorectal cancer, it would be of great interest to understand how Cdx2 inhibits cell migration and metastasis formation to improve prognosis or develop targeted therapy against metastatic cells. Further studies are required to decipher the exact mechanisms (new target genes, other non-transcriptional effects) of Cdx2 function.*

6. Cdx2 in other pathologies

6.1. Cdx2 in intestinal metaplasia

The replacement of the normal epithelium of the stomach or esophagus by an intestinal-like epithelium is called intestinal metaplasia. Metaplasia are not neoplastic by themselves but are considered to be precancerous lesions, because an important fraction of them leads to cancer. The findings obtained in transgenic mice (see III)3.3.2, page 39) indicate that gain of Cdx2 expression in the epithelium of the stomach or esophagus can induce an intestinal phenotype.

6.2. Cdx2 in inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are chronic inflammations of the intestine, among which ulcerative colitis and Crohn's disease are the most common. In Crohn's disease, transmural inflammatory lesions occur everywhere in the intestine but prevail in the terminal ileon, whereas in ulcerative colitis the lesions are limited to the colon (Coskun et al., 2011). Patients suffering from IBD present symptoms like diarrhea and abdominal pain and have an increased risk to develop colorectal cancer (Ballinger and Anggiansah, 2007). Several studies suggest the involvement of Cdx2 in IBD. Indeed, Cdx2 expression is down-regulated *in vitro* by several pro inflammatory factors such as the cytokines TNF- α (Kim et al., 2002, Cocksun 2012). In addition, Cdx2 inhibited the expression of COX-2, which encodes a proinflammatory enzyme (Kim et al., 2004). *In vivo*, Cdx2^{+/-} mice were hypersensitive to acute DSS-induced inflammation, probably because of their higher barrier permeability. However, these mice recovered faster than their wild-type littermates, potentially

because Cdx2 inhibited proliferation and migration, which are required for intestinal restitution. Paradoxically, upon chronic DSS treatment, the Cdx2^{+/-} mice developed less colon tumors, which can be explained by the reduced duration of inflammation (Calon, Gross, et al., 2007). Nevertheless, according to a recent study, Cdx2 expression seemed to be normal in tissues from a small cohort of IBD patients (Laurent et al., 2011) and thus determining the implication and role of Cdx2 in IBD will require more investigations.

6.3. Cdx2 in non-intestinal solid tumors

Ectopic Cdx2 expression can occur in all tumors of the gastrointestinal tract, especially in gastric and esophageal cancer. Cdx2 expression is very rare in other solid tumors with the exception of adenocarcinomas of the ovary for which Cdx2 is detected in 20% of the tumors (Moskaluk et al., 2003; Kaimaktchiev et al., 2004). Similarly to colorectal cancer, Cdx2 expression decreases in gastric tumors during progression and is inversely correlated with lymph node invasion (Fan et al., 2005; Xie et al., 2010; Okayama et al., 2009).

6.4. Cdx2 in leukemia

Unexpectedly, 90% of acute myeloid leukemia (AML) patients exhibit ectopic Cdx2 expression (Scholl et al., 2007). Cdx2 expression is a marker of bad prognosis and reduced survival for leukemia patients (Thoene et al., 2009). In contrast to intestinal cells, Cdx2 has oncogenic potential in hematopoietic cells: indeed, the knockdown of Cdx2 expression in leukemia cell lines inhibited their proliferation and their ability to form colonies *in vitro* (Scholl et al., 2007). Furthermore, ectopic Cdx2 expression in transplanted hematopoietic cells was sufficient to induce AML in mice (Rawat et al., 2004), by perturbing the expression of HOX genes (Rawat et al., 2008; Scholl et al., 2007). A potential explanation for the opposing effects observed in colon and leukemia cells is that Cdx2 interacts with different partners in these two cell types and thereby induces different transcriptomic programs and/or different cellular effects. Renouf et al gave the first proofs for this hypothesis as they showed that, in contrast to colon cancer cells, Cdx2 does not interact with and inhibit the DNA repair complex Ku70/Ku80 in leukemia cells. Consistently, upon genotoxic stress, Cdx2 expression inhibited cell survival in intestinal cells but rather enhanced cell recovery of potentially damaged leukemia cells (Renouf et al., 2012).

In summary, depending on the context, Cdx2 expression seems to impact very differently on tumorigenesis. In the intestine, reduced expression of Cdx2 seems to enhance CRC, whereas ectopic expression of Cdx2 in hematopoietic or gastric cells may be oncogenic. The mechanisms involved in the different contexts are not yet understood.

IV) Cadherins, a superfamily of adhesion molecules critical for intestinal homeostasis

1. Intestinal adhesion complexes

In the intestine, intercellular adhesion molecules are crucial for the integrity of the epithelium and its barrier function, but are also important mediators of cell signaling. These molecules are concentrated in adhesion complexes whose localization exhibit apico/basal polarity. On the apical side of the cells, the **tight junctions** (zonula occludens) seal the epithelium and at the same time regulate the passive solute flow (ions and small water-soluble molecules) across the intestinal epithelial barrier (Fasano, 2011). **Adherens junctions** anchor adjacent cells through their cytoplasmic actin filaments and **desmosomes** link the cells mechanically (**Figure 13**) (Groschwitz and Hogan, 2009).

Among these adhesion complexes, adherens junctions play a prominent role in cell polarity and tissue architecture. Members of the cadherin superfamily are key functional components of adherens junctions and are linked via intra-cellular catenins to the actin cytoskeleton (Angst et al., 2001).

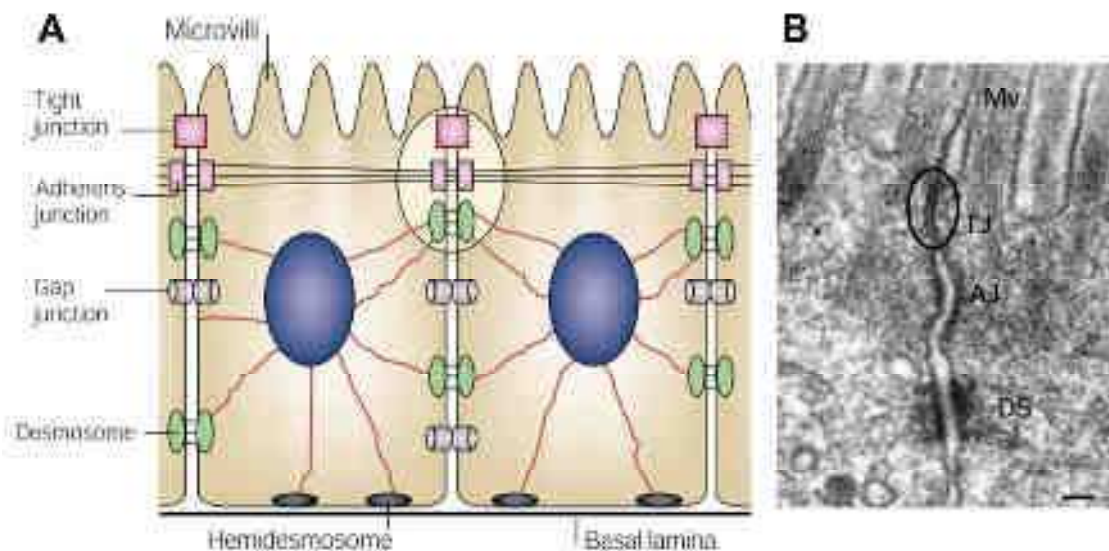


Figure 13: Adhesion protein complexes in the intestinal epithelium

(A) Schematic drawing and (B) Electron microscopy image of the protein complexes in the intestinal epithelium. Tight junction (TJ), adherens junction (AJ), desmosome (DS), microvilli (Mv) (Tsukita et al., 2001).

2. The cadherin superfamily

The cadherin superfamily comprises a large number (>100) of structurally very different proteins. Nevertheless, all cadherins are membranous glycoproteins that exhibit typical cadherin tandem repeats (ECs) on their ectodomain. These cadherin repeats have a length of about 110 amino acids and contain the negatively charged DXD, DRE, and DXNDNAPXF sequence motifs, which are involved in Ca^{2+} binding. However, the number and sequence of cadherin repeats can vary between the different cadherin family members (**Figure 15**).

2.1.1. Classical cadherins

Classical cadherins mediate Ca^{2+} -dependent cell-cell adhesion via their 5 cadherin repeats on their extracellular domain and primarily engage in homophilic interactions (**Figure 14**) (Angst et al., 2001; Pokutta and Weis, 2007). The best-described classical cadherin is E-cadherin that constitutes the transmembrane part of the adherens junction in epithelial cells, including intestinal cells. Other members of this sub-group are N-cadherin and Cadherin 11, which are expressed in mesenchymal cells, and VE-cadherin that is specific to endothelial cells (Wheelock et al., 2008). Their cytoplasmic domain is highly conserved and directly interacts with β -catenin, which links the cadherin to the actin cytoskeleton through α -catenin binding (**Figure 15**). Another direct interaction partner of classical cadherins is the catenin p120 which modulates the stability of cadherin-mediated cell-cell adhesion (Angst et al., 2001; Pokutta and Weis, 2007).

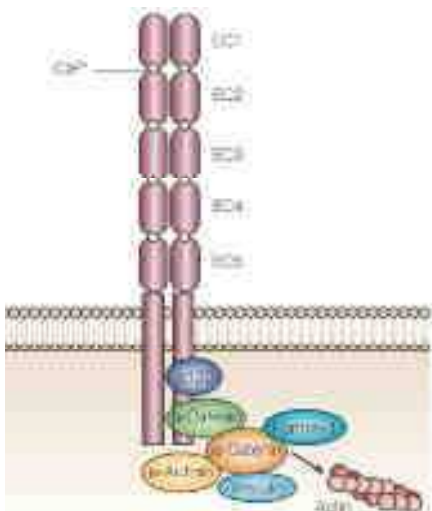


Figure 14: Structure of classical cadherins

Classical cadherins are composed of 5 extracellular cadherin repeats (ECs), a single pass transmembrane region and a cytoplasmic domain that interacts with partners such as p120 and β -catenin. The intracellular domain is linked to the actin cytoskeleton via β -catenin and other connecting proteins (Gumbiner, 2005).

2.1.2. Desmosomal cadherins

Two types of desmosomal cadherins, the desmogleins (Dsg) and the desmocollins (Dsc), constitute the transmembrane part of desmosomes. While the structure of their extracellular domain is similar to that of classical cadherins, their cytoplasmic domain is longer and very different. This domain is linked to the cellular cytoskeleton through members of the armadillo family (plakoglobin, plakophilin) and of the plakin family. They interact in a homophilic/heterophilic fashion (Angst et al., 2001). In particular, Dsc2 and Dsg2 are expressed in the intestinal epithelium, where they contribute to barrier function.

2.1.3. Protocadherins

They constitute by far the largest sub-group of cadherins in mammals. Since protocadherins are also expressed in some non-vertebrates, they may correspond to the ancestral cadherins. In mammals, expression of most protocadherins is specific to the nervous system (Angst et al., 2001), but intestinal cells have been shown to express the protocadherin Pcdh24 (LKC) for instance (Okazaki et al., 2002). Within this sub-group, the number of cadherin repeats is variable, but generally high, and the sequence of the repeats shows limited homology with those of classical cadherins. Protocadherins generally do not interact with catenins, as their cytoplasmic domains are most of the time also very different from the ones of classical cadherins (Halbleib and Nelson, 2006; Yagi and Takeichi, 2000).

2.1.4. Atypical cadherins

Cadherins like LI-cadherin and T-cadherin (the only known GPI-anchored cadherin) have cadherin repeats and can mediate cell adhesion, but, having no cytoplasmic domain, do not interact with the cytoskeleton (Gumbiner, 2005). Of note, LI-Cadherin is expressed in differentiated intestinal cells and was proposed to serve as a Ca^{2+} -regulated switch for the adhesive system on basolateral membranes of the intestinal epithelium: indeed, its binding responds to very small changes in extracellular Ca^{2+} (Wendeler et al., 2007).

2.1.5. Cadherin related proteins

This sub-group comprises very peculiar cadherins like the FAT cadherin which has 34 EC repeats, the Flamingo cadherin that has a 7 pass transmembrane region or Dachshous with 27 EC repeats. They are involved in embryonic patterning and planar cell polarity, but their role in cell adhesion has not been established (Gumbiner, 2005; Angst et al., 2001).

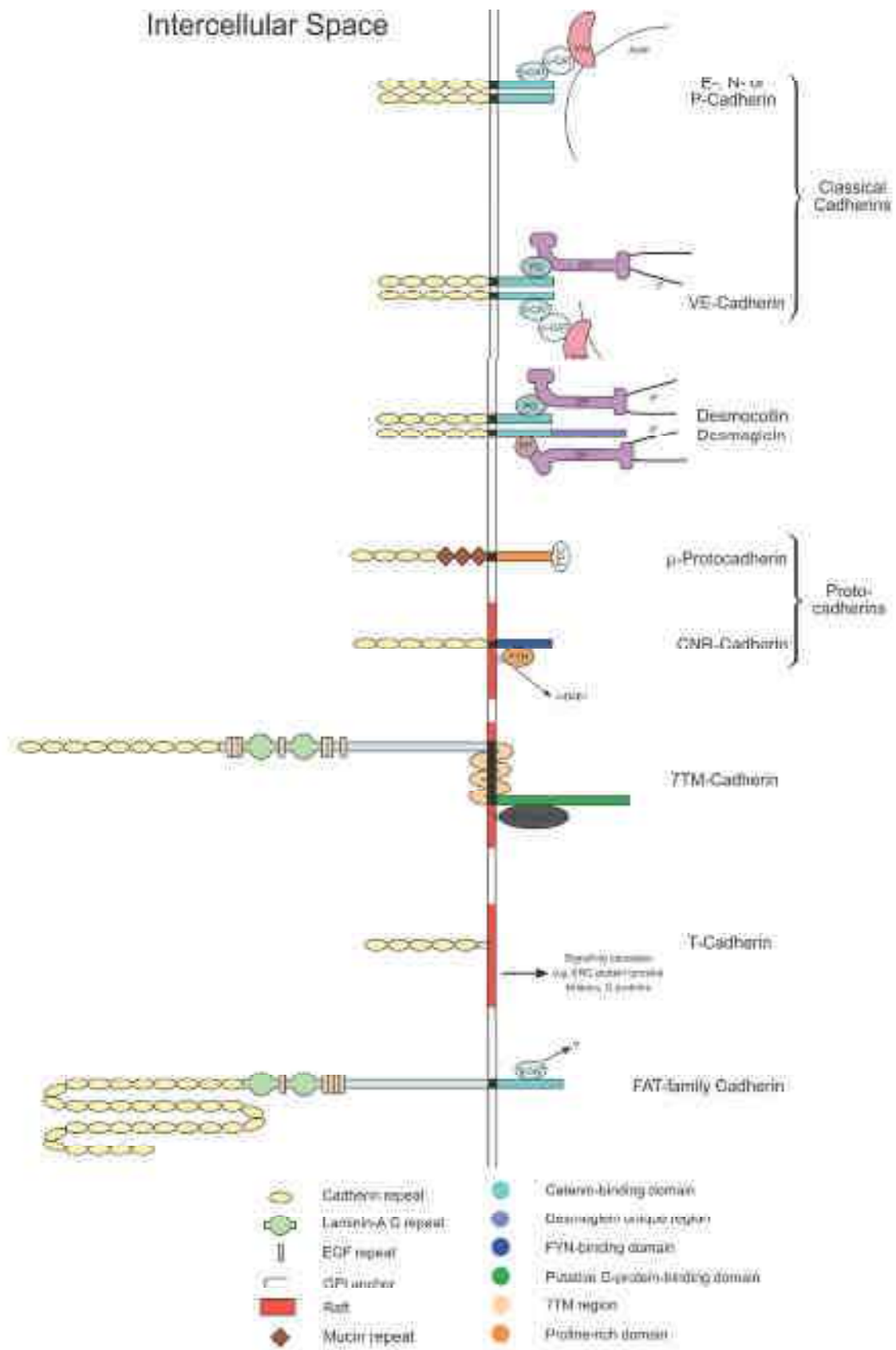


Figure 15: Overview of the diversity of the cadherin family

Cadherins expose a diversity of forms and functions having in common the presence of cadherin repeats in their extracellular domain (Angst et al., 2001).

3. Roles of cadherins

Because of the huge structural and functional diversity of cadherins, there is a multitude of pathways and cellular processes regulated by these adhesion molecules. At the moment, the classical cadherins are the most studied, but raising evidence shows that atypical cadherins and protocadherins also play important roles in embryonic development, tissue homeostasis and cancer initiation/progression.

The function of cadherins is not limited to simple cell-cell adhesion, as they are also involved in cell sorting, cell recognition, cell signaling, cell communication, morphogenesis, contact inhibition, cell growth and the induction / maintenance of cell or tissue polarity (Angst et al., 2001). For instance, during embryonic development, they mediate the association of single cells and regulation of cell sorting that gives rise to single cell sheets. In the adult intestinal epithelium, cadherins are important for tissue integrity and polarization of the intestinal epithelium (Wheelock and Johnson, 2003).

In addition, E-cadherin interacts with β -catenin at the cell membrane, the main effector of the Wnt signaling pathway and thus has the potential to impact this pathway critical for intestinal cells. Whether E-cadherin expression prevents β -catenin nuclear activity by sequestering it at the membrane is still a source of debate: the loss of E-cadherin expression does not automatically increase the cytoplasmic pool of β -catenin, but introduction of E-cadherin in a E-cadherin-deficient cell line can segregate β -catenin to the cell membrane (Hajra and Fearon, 2002). It has also been suggested that E-cadherin promotes the formation of a β -catenin destruction complex at the cell membrane (Maher et al., 2009). Anyway, in mice the specific loss of E-cadherin expression in the adult intestinal epithelium led to hemorrhagic diarrhea and rapid death. The intestinal architecture of the conditional Knockout mice was severely altered with the disappearance of adherent junctions and abnormalities in maturation or positioning of goblet cells and Paneth cells. Moreover, the clearance of enteropathogenic bacteria from the lumen was perturbed (Schneider et al., 2010).

Of note, cadherins exert their functions by interacting with other cadherins on neighboring cells, but interactions with other cell surface receptors have also been described. For instance, N-cadherin can interact with FGFR, which increases the abundance of this receptor at the cell surface by inhibiting its internalization and also stimulates FGF-dependent signaling by enhancing its dimerization (Cavallaro and Dejana, 2011). In contrast, E-cadherin can inhibit FGF signaling by affecting the trafficking and function of FGFR1 (Bryant et al., 2005).

4. Cadherins and cancer

4.1. Modifications of cadherins in cancer

The expression of adhesion molecules is often deregulated in cancer cells, with some molecules being down-regulated and others being ectopically expressed, which can lead to pro- or anti-oncogenic effects depending on the cell type and context.

Concerning E-cadherin, the gene is rarely mutated in colon cancer but can be silenced by epigenetic modifications (hypermethylation) or transcriptional regulation. For instance, a cadherin switch (replacement of epithelial E-cadherin by mesenchymal N-cadherin) has been observed in cells from the invasive front and probably undergoing tumoral EMT in colon and other cancers (Prall, 2007; Brabletz et al., 2005a). The switch seems to be mediated by the accumulation in these cells of transcriptional repressors of E-cadherin, such as Slug or Zeb1, in response to deregulated expression of miRNA (Brabletz and Brabletz, 2010). Remarkably, E-cadherin is often detected on cells of the metastases, suggesting a dynamic process. Additional mechanisms of E-cadherin protein level reduction in tumors that have been reported are: intracellular proteolysis, extracellular cleavage of E-cadherin and endocytosis (Christofori, 2006).

Other cadherins also have their expression modified in colon cancer cells. For instance, protein levels of Pcdh24, Dsc2 and T-cadherin are decreased, while the ones of P-cadherin are increased during tumor progression (Sun et al., 2011; Kolegraff et al., 2011; Okazaki et al., 2002; Ren and Huo, 2012).

4.2. Consequences of cadherin alterations in CRC cells

Given the role played by cadherins in important cellular processes such as cell adhesion or proliferation, modification of their expression can obviously impact tumor progression. The EMT cadherin switch described in invasive colon cancer cells represents a good illustration of the consequences of cadherin alteration. Indeed, absence of E-cadherin expression in tumor cells leads to reduced mechanical cell-cell adhesion, provoking the loss of polarity and tissue architecture, but also alteration of specific cell signaling pathways, which correlates with a more invasive phenotype: in particular, the loss of E-cadherin in colon cancer cells that are already deficient for the APC destruction complex, favors nuclear accumulation of β -catenin and transcriptional stimulation of pro-invasive genes (Hajra and Fearon, 2002; Gumbiner, 2005). However, the loss of one E-cadherin allele in double heterozygous $APC^{(+/1638N)}/E-cad^{(+/-)}$ animals, while clearly enhancing APC-driven tumor initiation (9-fold increase of tumor number), did not stimulate tumor progression (equivalent grade and stage of the tumors), suggesting that reduction of E-cadherin may not be enough (Smits et al.,

2000). At the same time, the gain of N-cadherin function renders tumor cells more aggressive: indeed, beside its direct effect on cell motility, N-cadherin also facilitates the contact of cancer cells and endothelial cells, thereby facilitating their dissemination through blood vessels (Hajra and Fearon, 2002; Wheelock et al., 2008).

Modification of expression of atypical cadherins and protocadherins may also play a role in colon cancer progression. For instance, the decrease of Pcdh24 observed in colon tumors leads *in vitro* to the loss of contact inhibition by reducing β -catenin activity through an yet unknown mechanism (Ose et al., 2009). The loss of Dsc2 promotes cell proliferation and enables colon tumor growth in nude mice through the activation of Akt/ β -catenin signaling (Kolegraff et al., 2011). In contrast, the up-regulation of P-cadherin in liver metastasis seems to promote proliferation and colony formation, possibly through stimulation of β -catenin transcriptional activity (Sun et al., 2011).

In summary, adhesion molecules and especially the very diverse family of cadherins are not only important for cell adhesion and tissue integrity, but are also involved in multiple signaling pathways. Indeed, they have a strong impact on cell polarization, proliferation, migration, apoptosis and migration as they can interact with various intracellular (catenins) and transmembrane (growth factor receptors, other cadherins) partners. Consequently, the modification of their expression has important consequences for tissue homeostasis, cancer initiation/progression and metastasis formation.

OBJECTIVES AND OVERVIEW OF THE PHD PROJECTS

Objective 1

In Europe, colorectal cancer is a leading cause of cancer-related death and represents a major problem for public health. The mortality of colorectal cancer is mainly due to resistance to chemotherapy and to the formation of metastases in the liver and lung. These two processes are not well understood at the moment, but may be linked to the molecular and cellular heterogeneity of tumors. Indeed, metastasis formation is not only driven by permanent genomic alterations, but also transient modifications of gene expression, that affect the morphology and function of cancer cells. Importantly, this plasticity may enable the cells to engage the important steps of invasion and migration to distant sites. For instance, disseminating colon cancer cells show a transient decrease in Cdx2 expression, which favors migration and invasion *in vitro*, and forced expression of Cdx2 limits the incidence of metastasis in nude mice.

Because these modifications are reversible, they represent potential targets for cancer therapy. However, as metastasis formation is a highly dynamic process that is influenced by multiple factors *in vivo*, **no appropriate experimental model exists at the moment that permits to integrate the concept of plasticity and really understand its role in the process of metastasis formation.**

→ **Objective: Determine the influence of cellular plasticity on tumor progression.**

PROJECT 1:

Tumor progression and plasticity:

Generation and characterization of a tumor heterogeneity model

My aim was to elucidate the influence of the tumor plasticity on colon cancer progression by generating a Cdx2 reporter cell line that reflects the heterogeneity of cancer cells, and permits to segregate disseminating, non-differentiated, tumor cells from differentiated tumor cells. These cell populations will be analyzed on the molecular and functional level.

Objective 2

The intestinal transcription factor Cdx2 can act as tumor and metastasis suppressor in the colon. Many target genes of Cdx2 have been identified, most of them being implicated in intestinal cell differentiation. However, the molecular relays of the anti-oncogenic effects of Cdx2 are not well defined. The systematic and transient decrease of Cdx2 expression in invasive cells at the tumor front suggests **Cdx2 might be an important actor in the tumor cell plasticity involved in metastasis.**

→ **Objective: Identify and characterize new molecular relays of Cdx2 in colon cancer cells.**

1st approach: From function to target genes

PROJECT 2:

**Molecular relays of Cdx2: study of their regulation and implication in Cdx2 function:
Cdx2 opposes elasticity of colon cancer cells through reorganization of the actin cytoskeleton
and enhanced cell-cell contacts**

This work started as a collaborative project with D. Vautier (INSERM U977, Strasbourg). The objective was to determine the impact of Cdx2 on cellular stiffness using atomic force microscopy, as it has been shown that the cells' elasticity correlates with a more invasive behavior (Suresh, 2007). My part of the work was the identification of Cdx2 target genes that could mediate this effect.

2nd approach: From target gene to function

PROJECT 3:

**Molecular relays of Cdx2: study of their regulation and implication in Cdx2 function:
Mucdhl, a new player in intestinal homeostasis and colorectal cancer progression?**

Comparison of various transcriptomes obtained through microarray-based screens allowed the identification of potential Cdx2 target genes, including Mucdhl, a gene whose regulation and function was not known. My first aim of this project was to determine if Mucdhl was a direct target gene of Cdx2. The second aim was to elucidate the function of Mucdhl in intestinal cancer cells.

PROJECT 1:

Tumor progression and plasticity

Generation and characterization of a tumor heterogeneity model

“The physicist's problem is the problem of ultimate origins and ultimate natural laws. The biologist's problem is the problem of complexity.”

Richard Dawkins
Evolutionary biologist and author

Introduction

1. Context

Colorectal cancer is a major public health problem as it is the fourth leading cause of cancer death and its incidence is increasing worldwide. The important mortality of this cancer, especially in the later stages, is caused by metastases that occur primarily in the liver and its propensity to resist to chemotherapy (Markowitz et al., 2002; Ballinger and Anggiansah, 2007). At the moment, both processes are only poorly understood.

Colorectal tumors present an important intra-tumoral heterogeneity, as in the same tumor cell populations with different molecular alterations, differentiation grades, proliferative activity and aggressiveness co-exist. Moreover, tumor heterogeneity may not only occur in space, but also in time (Brabletz et al., 2004). Indeed, in the last years, it was proposed that transient modifications of cancer cells might have an important impact on tumor progression. In contrast to cancers cells located in the center of the primary tumor or the metastasis, disseminating cancer cells located at the tumor front do not exhibit a typical epithelial phenotype, suggesting that they have undergone tumoral EMT (dedifferentiation of epithelial cancer cells that gain mesenchymal properties) and/or have characteristics of cancer stem cells (Mani et al., 2008; Thiery et al., 2009) (see II)2.4.3, page 31). In particular, the expression of important actors of intestinal homeostasis is transiently altered in disseminating cancer cells: expression of E-cadherin and Cdx2 is reduced, whereas nuclear localization of β -catenin is enhanced. Importantly, the micro-environment may be conditioning most of these transient modifications of protein expression or activity.

Thus, the molecular and cellular heterogeneity of tumors, conditioned by permanent genomic alterations and transient regulations depending of the micro-environment, might reflect the cellular plasticity of cancer cells and enable metastasis. Because metastasis formation is a highly dynamic process, which takes a long time, and is sensitive to the micro-environment, it seems difficult to recapitulate it *in vitro*. Moreover, no adequate experimental model existed at the beginning of this project to study the importance of tumoral heterogeneity and cellular plasticity for this process.

2. Aim

Cdx2 can be considered as a heterogeneity marker in colon cancer. I aimed at developing a model to study the importance of tumor heterogeneity for metastasis formation. To do so, I created a reporter cell line of Cdx2 expression that if further validated will permit the separation of different tumor cell populations by the means of Cdx2 expression levels.

3. Experimental approach

3.1 Bases of the model

To create this reporter system, I decided to take advantage of two colon cancer cell lines that model the plasticity of Cdx2 expression. T84 cells exhibit strong expression of Cdx2 *in vitro*, but lose it when they are grafted into the cecum wall. The mesenchymal environment in the cecum wall represses Cdx2 expression, so this model recapitulates the situation in invasive colon cancer cells, which lose Cdx2 expression when they are in the vicinity of mesenchymal cells. On the other hand, HT29 cells do not express Cdx2 *in vitro*: nevertheless, they gain Cdx2 expression when grafted under the skin of nude mice (Benahmed et al., 2007). In consequence, the injection site under the skin mimics the situation in metastasis, in which colon cancer cells express Cdx2 (Brabletz et al., 2004). It is important to note that, as the HT29 and T84 lines are genetically stable, only the reversible changes induced by the micro-environment should impact the tumor progression. Thus, these two cell lines will be adequate to create the Cdx2 reporter cell lines.

3.2 Generation of the Cdx2 reporter cell line

The Cdx2 reporter cell lines will permit to separate different tumor cell populations by the means of Cdx2 expression levels. To create these cell lines, HT29 and T84 cells will first be stably transfected with the mCherry-N1 plasmid, which expresses a red fluorescent protein that will serve to separate murine and human cells in xenografting experiments. In a second step, the coding sequence of the green fluorescent protein (GFP) will be introduced in the region of the Cdx2 locus (**Figure 1**). This GFP sequence will be in the same reading frame than Cdx2, resulting in the translation of a Cdx2-GFP fusion protein. In consequence, the expression level of Cdx2 will be reflected by the intensity of the green fluorescence of the cells. Of note, a simpler strategy in which the GFP coding sequence would have been placed under the control of a Cdx2 promoter fragment could have been proposed: however, previous attempts of generating a Cdx2 reporter cell line in the lab suggested that the promoter fragments were not able to recapitulate

endogenous expression of Cdx2 (I. Duluc, personal communication).

The GFP coding sequence will be introduced into the locus of Cdx2 by somatic homologous recombination. The limiting steps in human gene targeting by homologous recombination are gene delivery and integration. I chose to use rAAVs (recombinant Adeno associated viruses) to deliver the homologous recombination cassette into the cells because it has been shown that gene targeting with rAAVs increases the number of positive targeting events up to 25 times compared to transfected plasmids that target the same exon (Topaloglu et al., 2005). Importantly, successful recombinant recombination was already reported in the HT29 cell line (Montrose-Rafizadeh et al., 1997; Dang et al., 2006) and, although some cell lines are difficult to transduce with AAVs, the HT29 and T84 cell lines were already validated for gene delivery with rAAVs.

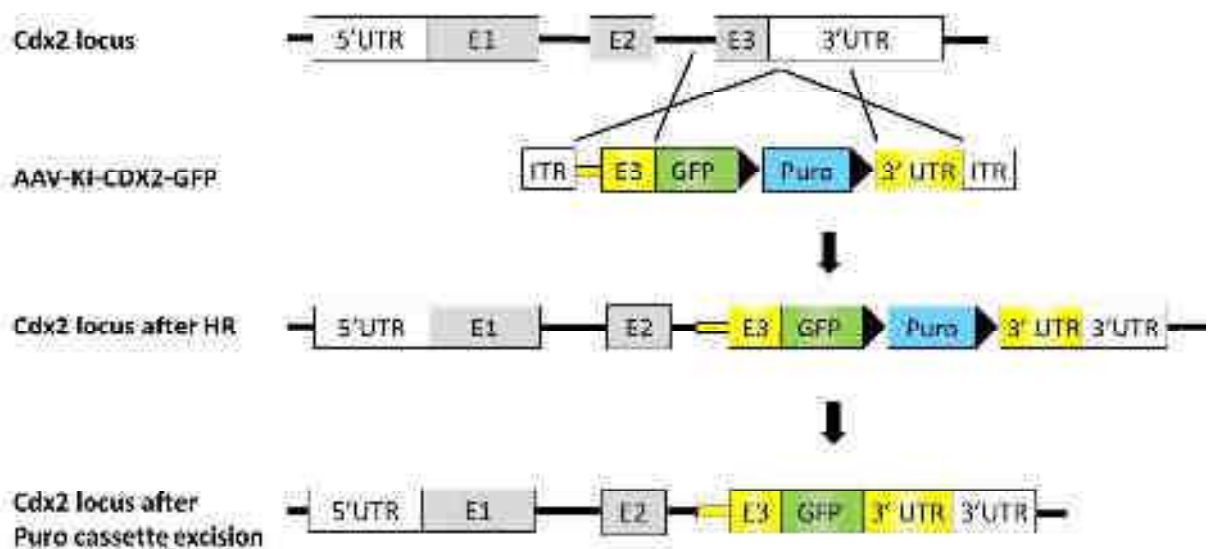


Figure 1: Strategy to insert the coding sequence of GFP into the Cdx2 locus

In cells infected with viral particles carrying the AAV-KI-Cdx2-GFP targeting construct, homologous recombination through homology arms (yellow) can occur at the Cdx2 locus. Next, the puromycin resistance cassette can be excised by a Cre recombinase. E: exon; UTR: untranslated region; ITR: inverted terminal repeat; HR: homologous recombination.

3.3 Generation and analysis of different cell populations

To study the impact of cell plasticity on tumor progression, the reporter cell lines will be xenografted into nude mice (**Figure 2**). The tumors formed will be harvested and the cells with different fluorescence levels will be sorted by flow cytometry. The red fluorescence of the injected cells will permit to distinguish human and murine cells and the green fluorescence will

separate Cdx2 expressing and non-expressing cells. In this way, 4 different cell populations will be obtained: T84 Cdx2^{high}, T84 Cdx2^{low}, HT29 Cdx2^{high} and HT29 Cdx2^{low}. These cell populations will at first be characterized on the molecular level (comparison of their transcriptomes by DNA microarray) and next on the functional level by assessing their properties, for instance their proliferation and aggressiveness (xenografts to nude mice).

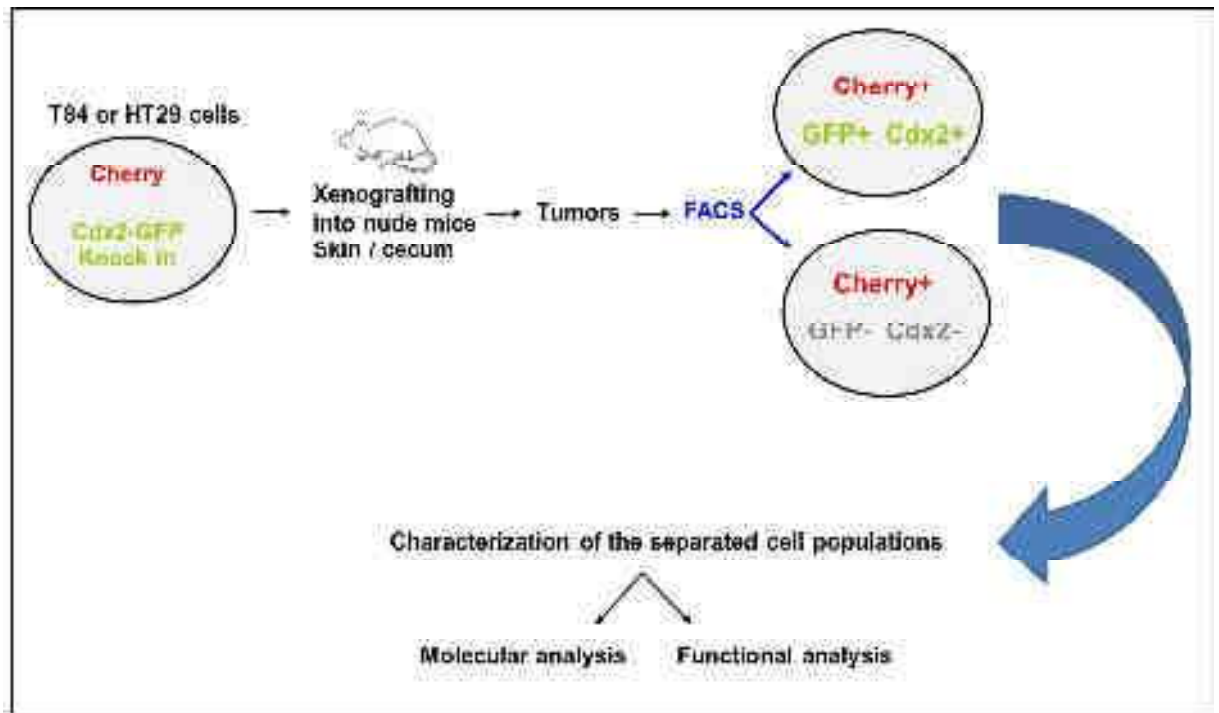


Figure 2 : Schematic overview of the project

Step1: Generation of the Cdx2 reporter cell lines.

Step2: Injection of these cells under the skin or into the cecum wall of nude mice.

Step3: Harvest of the tumors and separation of the cells by FACS.

Step4: Characterization of the cells.

Materials and Methods

Primers

| Primer name | 5'-3' sequence |
|-------------|---|
| SHANotS-F | TGAGCGGCCGCGACAAGACTTGGTTCATGAACAGAGGGATATAAGAAAGTGTGAA |
| SHANotS-R | AAGCTTAAGCTGGGTGACGGTGGGGTTTAG |
| GFP-All | GTAAGCTTAAGGTGAGCAAGGGCGAGGAGCTGTTC |
| GFP-LV | TALGAAATCAACTAGAAAGCACTGGAAAAAAGCTT |
| mutC2Afl-F | CACCTTCACCCAGCTTAAGACCCGGTTCCTGGAGCCGG |
| mutC2Afl-R | CCGCTGCAGAACCCGGTCTTAAGCTGGGTGACGGTGG |
| SHAGFP-B4 | GGGGACAACCTTTGTATAGAAAAGTTGTGAGCGGCCGCTAGCTGGGACT |
| SHAGFP-B1r | GGGGACTGCTTTTTGTACAAACTTGGATATCAACTAGAATGCAGTG |
| 3HA-B2r | GGGGACAGCTTTCTTGTACAAAGTGGCCACCAGGTTCTGCAGCGGCA |
| 3HANot-B3 | GGGGACAACCTTTGTATAATAAAGTTGGCGCCCGCCACTTCCCTTCACCATATCA |
| 1P-B1 | GGGGACAAGCTTTGTAAACAAAAAGCAGCCCTGTTTCACTTAAACCGTAIAACTT |
| PE-B2 | GGGGACCACCTTTGTACAAGAAAGCTGGGTGCTAGTCGACTTCGAATAACTT |
| Ctrl 5' F | CTTTTGTCCACATGTAGATGGGGTCTTGTCTTTGTG |
| Ctrl 5' R | GCCTGCAGGACATGTAAGAGGACTAAAGGTAGAAG |
| Screen F | TAGCTGGGACTACAGGCAT |
| Screen R | TGTTGATGAAACCAAGTCTTGTGTC |
| 5'HA -F3 | GCTCTCTTCTACCTTTAGTCT |
| 5'HA -R3 | TTCAGGGTCAGCTTGGCGTA |
| 3'HA -F4 | ATA GGG GTT CCG CGC ACA TTT C |
| 3'HA -R4 | CAG AAT CTG AAA TCT GGA AAG CTC |

Plasmids

- pHelper, AAV-RC and pAAV-MCS (AAV Helper-Free System, Stratagene)
- pDONR P4-P1R, pDONR 221, pDONR P2R-P3, pDEST R4-R3 (MultiSite Gateway Three-Fragment Vector Construction Kit, Invitrogen)
- pcR11/TOPO (Invitrogen)
- pEGFP-C1 and pmCherry-N1 (Clontech)

Cell lines

Human colon adenocarcinoma cell lines HT29 and T84 (ATCC) were respectively maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) and Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Invitrogen), and antibiotics.

- HT29-Cherry selection media: HT29 media supplemented with 1.4 mg/ml G418 (Invitrogen)
- T84-Cherry selection media: T84 media supplemented with 1 mg/ml G418
- HT29-**CDX2-GFP** media: HT29 media supplemented with 1.4 mg/ml G418 and 0.7 µg/ml puromycin (Invitrogen).

The packaging cell line AAV-Hek293 (AAV Helper-Free System, Stratagene) and human colon cancer HCT116 cells (ATCC) were cultivated in DMEM, supplemented with 10% FBS and antibiotics.

Cloning of the pEntry vectors

For an overview of the complete cloning strategy see **Figure 3** p. 74

TOPO-GFPpA

pEGFP-C1 (Clontech) was used as a template to amplify by PCR a fragment coding for EGFP followed by a polyadenylation signal (SV40 origin) with the Phusion polymerase (Finnzymes). The forward primer GFP-Afl added an AflIII restriction site on the 5' extremity and deleted the first Met of the EGFP. The reverse primer GFP-EV added an EcoRV restriction site on the 3' extremity. The PCR product AflIII-EGFPpA-EcoRV was sub-cloned into pcR11/TOPO by TA cloning (Invitrogen), generating TOPO-GFPpA.

5HA GFP pEntry

HT29 genomic DNA was used as a template to generate the left (5HA) homology arm for the Cdx2 gene targeting. PCR was performed with Platinum Pfx DNA Polymerase and PCRx enhancer solution (Invitrogen). The forward primer 5HANotS-F incorporated a NotI site on the 5' extremity and a sequence that serves as an annealing sequence for the reverse screening primer Screen R1. The reverse primer 5HAfl-R deleted the STOP codon of Cdx2 and added an AflIII restriction site on the 3' extremity. The PCR fragment NotI-5HA-Afl I was sub-cloned into pcR11/TOPO by TA cloning generating TOPO-5HA. The AflIII -EGFP-EcoRV fragment from TOPO-GFPpA was inserted between the AflIII and EcoRV sites of TOPO-5HA, generating TOPO-5HAGFPpA. This vector was used as a template to perform a PCR with the Platinum Pfx DNA Polymerase and PCRx enhancer

solution (Invitrogen) with primers 5HAGFP-B4 and 5HAGFP-B1r. The resulting PCR product, flanked with an attB4 site on the 5' extremity and an attB1r on the 3' extremity, was inserted by recombination with the BP clonase II mix (MultiSite Gateway Three-Fragment Vector Construction Kit, Invitrogen) into the plasmid pDONR P4-P1r, generating the attL4-HAGFPpA-attR1 entry plasmid (called 5HA-GFP pEntry).

PuroR pEntry

The plasmid PIGpuX1 (G. Mellitzer, U682) contains the coding sequence for the puromycin resistance gene under the control of a SV40 promoter flanked by loxP sites. The Platinum Pfx DNA Polymerase (Invitrogen) and the primers LP-B1 and PL-B2 were used to amplify this sequence. These primers generated a PCR product, flanked by the attB1 sequence on the 5' and an attB2 sequence on the 3' extremity, that was inserted into the vector pDONR 221 with the BP clonase II mix (MultiSite Gateway Three-Fragment Vector Construction Kit, Invitrogen) generating the attL1-Lox-Puro-Lox-attL2 entry plasmid (called PuroR pEntry).

3HA pEntry

HT29 genomic DNA was used as a template to generate the right (3HA) homology arm for the Cdx2 gene targeting. The Platinum Pfx DNA Polymerase (Invitrogen) and the primers LP-B1 and PL-B2 were used to amplify this sequence. The primers added an attB2r sequence on the 5' extremity and a NotI restriction site followed by an attB3 sequence on the 3' extremity. The PCR product was inserted into the vector pDONR P2R-P3 with the BP clonase II mix (MultiSite Gateway Three-Fragment Vector Construction Kit, Invitrogen) generating the attL2-3HA-attL3 entry plasmid (called 3HA pEntry).

Cloning of pAAV-KI-CDX2-GFP (viral knock-in vector)

The 3 PCR fragments that were first cloned into the entry vectors (5HA-GFP pEntry, PuroR pEntry, 3HA pEntry) were assembled in an orientated way into the pDest R3-R4 vector with the LR clonase II generating pDest-KI-**CDX2-GFP** (MultiSite Gateway Three-Fragment Vector Construction Kit, Invitrogen). pDest- KI **CDX2-GFP** was next digested with NotI and the fragment sub-cloned between the two NotI sites of pAAV-MCS to create the final gene targeting vector pAAV- KI-**CDX2-GFP**.

Cloning of pFlag-hCdx2-GFP (control vector for Cdx2-GFP activity)

pFLAG-hCdx2 was obtained by first sub-cloning a RT-PCR fragment containing the human Cdx2 CDS into pCRII/TOPO (Invitrogen) and next inserting a HindIII-XbaI fragment from the resulting plasmid into pFLAG-CMV2 (Sigma). By site-directed mutagenesis, using the QuikChange II XL Site-

Directed Mutagenesis Kit (Stratagene) and the primer pair mutC2Afl-F / mutC2Afl-R, the Cdx2 STOP codon was deleted by creation of an AflIII restriction site. TOPO-GFPpA was digested with Afl II-EcoRV and the released fragment was sub-cloned into AflIII-EcoRV of pFlag-hCdx2, inserting the GFPpA sequence in-frame with the Cdx2 coding sequence.

Control Cloning of pFlag-hCDX2 GFP (control vector for CDX2 GFP activity)

HT29 genomic DNA was used as a template to generate a 652 bp fragment of the region flanking the left homology arm using the primer pair Ctr 5 F/R. PCR was performed with Platinum Pfx DNA Polymerase and PCRx enhancer solution (Invitrogen). The plasmid pAAV-KI-CDX2-GFP was linearized with the restriction enzyme PciI and gel-purified. The PCR reaction added 15 bp extensions complementary to the vector ends, which permitted insertion of the PCR product into the vector with the In Fusion HD cloning enzyme (Clontech).

Production of AVV-Cdx2-GFP virus stock

To produce recombinant AAV particles containing the **CDX2-GFP** targeting cassette, 6×10^6 AAV-Hek293 cells were seeded in 75cm² flasks and transfected the next day with pAAV-KI-**CDX2-GFP**, AAV-RC and pHelper (AAV Helper-Free System, Agilent Technologies) using Lipofectamine 2000 (Invitrogen) as recommended. After 72 hours, cells were scraped in 1 ml cell of culture medium and lysed by 3 freeze-thaw cycles using a dry ice ethanol bath. Crude lysates were cleared by centrifugation at 10,000g for 10 minutes and the supernatants were stored at -80°C until use.

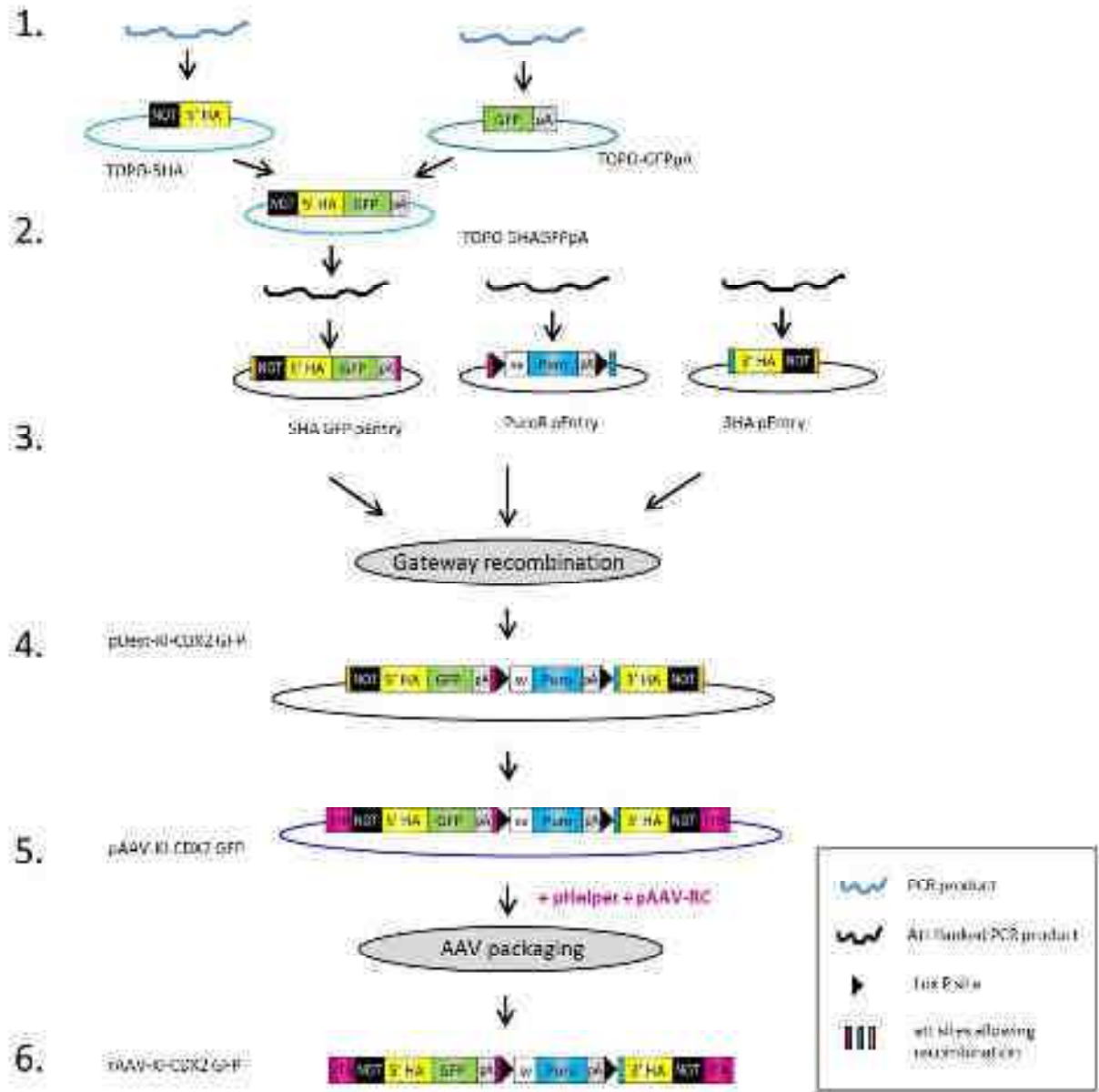


Figure 3 : Overview of the pAAV-KI-CDX2 GFP cloning strategy

In a first step (1.) the 5' homology arm and the GFP coding sequence were generated by PCR, cloned into a pcR11/TOPO vector and fused in a single vector (2.). The knock-in vector for pAAV-KI-CDX2-GFP was generated using the Gateway system (Invitrogen), which uses site-specific recombinational cloning via att sites to allow the assembly of 3 DNA fragments from 3 pEntry vectors into one destination vector (pDest) in a single step. To do this, PCR products corresponding to the 3 sequences of interest (puromycin resistance gene, left and right homology arms) were generated by PCR, inserted into the pEntry vectors (3.) and assembled by a recombinase in a single vector (4.). The targeting cassette was then cloned into the viral targeting vector by the NotI restriction sites (5.). In the pAAV-KI-CDX2-GFP vector, the knock-in cassette is flanked by two ITR (Inverted Terminal Repeats) sequences, responsible for viral packaging of the sequence (6.). All new constructs were checked by sequencing (GATC Biotech).

Generation and PCR identification of HT29-Cherry AAV cells

T84-Cherry and HT29-Cherry cells were established by transfection of the pmCherry-N1 vector with Lipofectamine 2000 (Invitrogen) as recommended and selection by G418 in T84/HT29-Cherry selection media.

After validation, 18×10^6 HT29-Cherry cells of the selected clone (HT29-Cherry-H4) were seeded in 75cm² flasks to be transduced one day later with 1ml of the virus stock diluted in 5 ml DMEM-2% FBS. After an incubation period of 4 hours at 37°C, 5 ml of DMEM-18% FBS were added. Stable clones were selected with puromycin by using the HT29-**CDX2-GFP** media and individually transferred to 96-well plates. When cells reached confluence, they were moved to a 24-well plate for amplification and a 96-well plate for PCR screening. Cells in the 96-well plate were lysed with Direct PCR Lysis Reagent (Viagen) supplemented with 0.4mg/ml proteinase K and incubated for 2 hours at 50°C and 45 min at 85°C. 1 µl of crude cell lysate was submitted to PCR using RedTaq master mix (Eurogentec) and products were analyzed on an Ethidium bromide agarose gel. The cycling conditions were 5min at 94°C, 35 cycles of 15sec at 94°C, 30sec (Screen R/F) or 90sec (5'HA-F3/R3, 3'HA F4/R4) at 58°C, 90sec at 72°C, and 1 final cycle of 3min at 72°C. A second pair of oligonucleotides (5'HA-F3/R3) was used to check the 5' integration, with the forward primer annealing outside of the left homology arm and the reverse primer annealing to the GFP coding sequence. To check 3' integration, a forward primer (3'HA-F4) annealing to the puromycin resistance gene and a reverse primer (3'HA-R4) annealing outside of the right homology arm were used.

Reverse transcription and qPCR

HCT116 cells were transfected with Lipofectamine 2000 (Invitrogen) as recommended. Total RNA was isolated using TRI Reagent (MRC). For RT-qPCR, reverse transcription (RT) was performed in 20 µl using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and 2 µg of RNA. Ten times diluted RT products were mixed with TaqMan Master Mix (Applied Biosystems) and gene-specific TaqMan probe and primers sets (TaqMan Gene Expression assays, Applied Biosystems) to perform quantitative PCR (qPCR) using the 7500 real-time PCR System (Applied Biosystems). The following TaqMan Gene Expression assays were used: Cdx2, Hs00230919_m1; LI-cadherin, Hs00184865_m1, MUC13, Hs00217230_m1; TBP, Hs99999910_m1). Duplicates of each experimental point were performed. Analyses of the results were done with the 7500 software v2.0.1 (Applied Biosystems) using the relative $\Delta\Delta C_t$ quantification method in which the fold corresponds to $2^{-\Delta\Delta C_t}$ with $\Delta\Delta C_t$ equals (Ct target gene – Ct endogenous control gene) – (Ct calibrator gene – Ct endogenous control gene).

Xenograft experiments

T84-Cherry or HT28-Cherry cells were injected subcutaneously (2×10^6 cells in 50 μ l / injection point) or into the cecum (1×10^6 million cells) of nude mice. Tumor volumes were measured weekly. After 8 weeks, mice were sacrificed and tumors were analyzed by immunohistochemistry.

Immunohistochemistry

For immunohistological studies of the grafted tumors, tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (6 μ m) were deparaffinized and heated for 10 min in 10 mM citrate buffer pH6.2 for antigen retrieval. They were stained with Harris solution for histological examination, or immunostained using the primary antibodies raised against Cdx2 (Clone 88, Biogenex, 1/200), a secondary biotinylated anti-mouse (Vector Laboratories Inc., dilution 1/200) and the Vectastain ABC kit (Vector Laboratories Inc) as recommended by the supplier. Cell nuclei were counterstained with H&E.

Western blot

Cells were lysed on ice for 10 min in Cytoplasmic lysis buffer (10 mM Tris pH 7.9, 15 mM NaCl, 10 mM $MgCl_2$, 0,1% NP-40 and one tablet of Complete MiniTM protease inhibitor cocktail (Roche Molecular Biochemicals) for 10 ml). After centrifugation at 800g for 5 minutes, the supernatants were collected and considered as the cytoplasmic fractions, while the nuclei were washed twice with Cytoplasmic lysis buffer before lysis in Nucleic lysis buffer (500 mM NaCl, 20mM Hepes pH 7.9, 20% glycerol and one tablet of Complete MiniTM protease inhibitor cocktail for 10 ml) and sonication. Protein concentrations were measured using Bio-Rad Protein Assay. 20 μ g of proteins were separated by SDS-PAGE and transferred to Immobilon-P nylon membranes (Millipore). Membranes were saturated at room temperature (RT) for 45 min in PBS containing 1 % BSA and incubated at 4 °C overnight with one of the following primary antibodies: M@Cdx2 (Biogenex; 1/5000), M@GFP (clone JL-8, Clontech; 1/1000), Rb@GAPDH (Marie Vanier Inserm U682; 1/3000) and Rb@phospho-histone H3 (PH3, Upstate; 1/3000). For the detection, peroxidase-conjugated sheep anti-mouse IgG antiserum or peroxidase-conjugated donkey anti-rabbit IgG antiserum (Amersham Pharmacia Biotech; 1/5000) was used at RT for 45 min. Finally, the membranes were developed using ECL (Amersham Pharmacia Biotech).

Results

1. Validation of the Cdx2-GFP fusion protein

The somatic homologous recombination will generate cells expressing a Cdx2-GFP fusion protein. To control that this chimeric protein would still be able to translocate to the nucleus and activate its target genes, a plasmid encoding the CDX2-GFP fusion protein was constructed.

After transfection of HCT116 cells with the plasmid encoding the wild-type protein or the fusion protein, cytoplasmic and nuclear protein fractions were extracted and analyzed by Western blot. Immunoblotting with antibodies against GAPDH and PH3 confirmed the purity of the cytoplasmic/nucleic fractions (**Figure 4A**). The antibody against Cdx2 detected both proteins (**Figure 4A**), whereas the anti-GFP antibody detected only the fusion protein (data not shown). Wild-type Cdx2 has a molecular weight of 42 kDa, and the band for CDX2-GFP was detected at 72 kDa, which corresponded to the addition of the molecular weight of Cdx2 and GFP (27 kDa). Like the wild-type protein, CDX2-GFP was mainly localized in the nucleus.

To verify that the presence of GFP at the C-terminus of Cdx2 did not inhibit target gene activation, wild-type Cdx2 and CDX2-GFP were overexpressed in HCT116 cells. Analysis of the expression levels of the Cdx2 target genes LI-cadherin and Muc13 by RT-qPCR showed that the fusion protein CDX2-GFP, even if slightly less efficient than wild-type Cdx2, was still able to activate target gene transcription (**Figure 4B**). Thus, the insertion of the GFP sequence in frame with the Cdx2 coding sequence by homologous recombination will probably not alter Cdx2 function.

2. Validation of HT29-Cherry and T84-Cherry stable cell lines

For each cell line, 13 G418-resistant clones with red fluorescence were generated. In order to choose the best clone to perform the homologous recombination, 3 T84-Cherry and 3 HT29-Cherry clones with very strong and homogeneous red fluorescence were chosen for further validation *in vivo* (**Figure 5A**). Their cells were injected under the skin or into the cecum wall of nude mice. After 8 weeks, all clones had formed tumors and all these tumors presented a strong and homogeneous red fluorescence (**Figure 5A**). To check that the cells exhibited the same heterogeneous Cdx2 expression pattern as the parental cell lines, immunohistochemical analyses of Cdx2 expression were performed. Cells that were injected subcutaneously gained (HT29-Cherry) or preserved Cdx2 expression (T84-Cherry) respectively (**Figure 5B**). Similarly, HT29-Cherry cells entering blood vessels in the cecum started to express Cdx2 in this new micro-

environment. In contrast, cells that were injected into the cecum wall lost Cdx2 expression (T84-Cherry) (**Figure 5B**). Taken together, these experiments indicated that the new T84-Cherry and HT29-Cherry stable cell lines were able to form red fluorescent tumors in which the Cdx2 expression was modulated by the micro-environment in the same way than in the parental cell lines.

3. Generation and identification of HT29-Cherry cells exhibiting homologous recombination at the Cdx2 locus

Because the generation and screening of two different types of stable cell lines by homologous recombination represents a lot of work, the knock-in construction was first only delivered to the selected HT29-Cherry clone. In the future, it will also be delivered to the T84-Cherry cells. HT29-Cherry cells were transduced with the recombinant AAVs containing the targeting cassette (rAAV-KI-CDX2-GFP) and puromycin resistant clones were selected and expanded.

Screening for clones with correct homologous recombination at the Cdx2 locus was performed by end-point PCR on genomic DNA of the cells. In a first PCR screen, 675 individual clones were analyzed with the ScreenF/R and 5'HA-F3/R3 primer pairs. The ScreenF/R primer pair was used to control the quality of genomic DNA extraction or PCR, and was predicted to amplify a fragment of similar size in negative and positives clones (0.4 kb) (**Figure 6A**). The 5'HA-F3/R3 primer pair was designed to generate a fragment of about 1.3 kb, only when the 5' extremity of the targeting cassette was integrated at the correct position in the Cdx2 locus. The forward primer recognizes a sequence in the second intron of Cdx2 outside of the homology region and the reverse primer anneals to a sequence in the coding sequence of GFP (**Figure 6A**). The problem with this primer pair is that no positive control for the PCR reaction would be available before the identification of the first correct recombinant clone, yielding some doubts in case of negative screening results. To circumvent this problem, a plasmid was constructed that contained a 632 bp fragment of the left homology region ahead of the knock-in cassette, mimicking the situation of a cell with a positive targeting event. This plasmid was used to optimize the PCR conditions for the 5'HA-F3/R3 primer pair and served as a positive control during the screening PCR reaction. Even so, out of the 675 clones that were analyzed, only one (number 198) was positive for the integration of the 5' extremity of the knock-in cassette (**Figure 6B**). A second PCR was performed on the genomic DNA of clone 198 to check if the 3' extremity was also correctly integrated into the 3'UTR of Cdx2, using the primer pair 3'HA-F4/R4. The

forward primer anneals to a sequence in the puromycin resistance gene and the reverse primer binds to a sequence outside of the homology region (**Figure 6A**). This primer pair generated a fragment at the expected size (about 1.3 kb) indicating that the targeting cassette integrated correctly into the Cdx2 locus (**Figure 6B**).

Figures

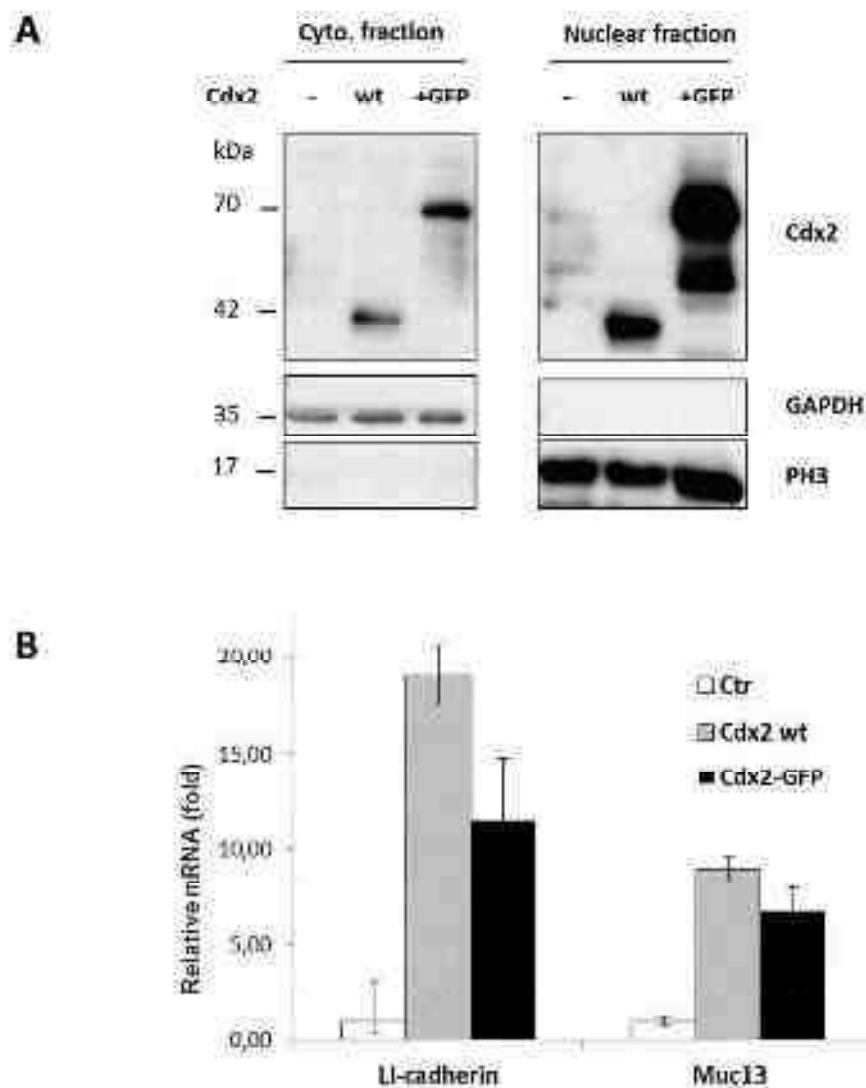


Figure 4: Validation of the CDX2-GFP fusion protein

(A) Western blot of cytoplasmic and nucleic extracts of HCT116 cells, transfected with a control plasmid (-), a plasmid coding for Cdx2 (wt) or the fusion protein CDX2-GFP (+GFP). Both proteins were detected with an anti-Cdx2 antibody. Detection of GAPDH (mostly cytoplasmic) served as loading control for the cytoplasmic fraction and PH3 (phospho-histone H3, chromatin bound) for the nuclear fraction. **(B)** The expression levels of two Cdx2 target genes (LI-cadherin and Muc13) were analyzed by RT-qPCR. This one was performed on RNA extracted from HCT116 cells transfected as detailed above in (A). Relative expression levels of the two target genes are presented as fold of induction, with the level (\pm standard deviation, normalized with TBP expression) in cells without Cdx2 overexpression set at 1.

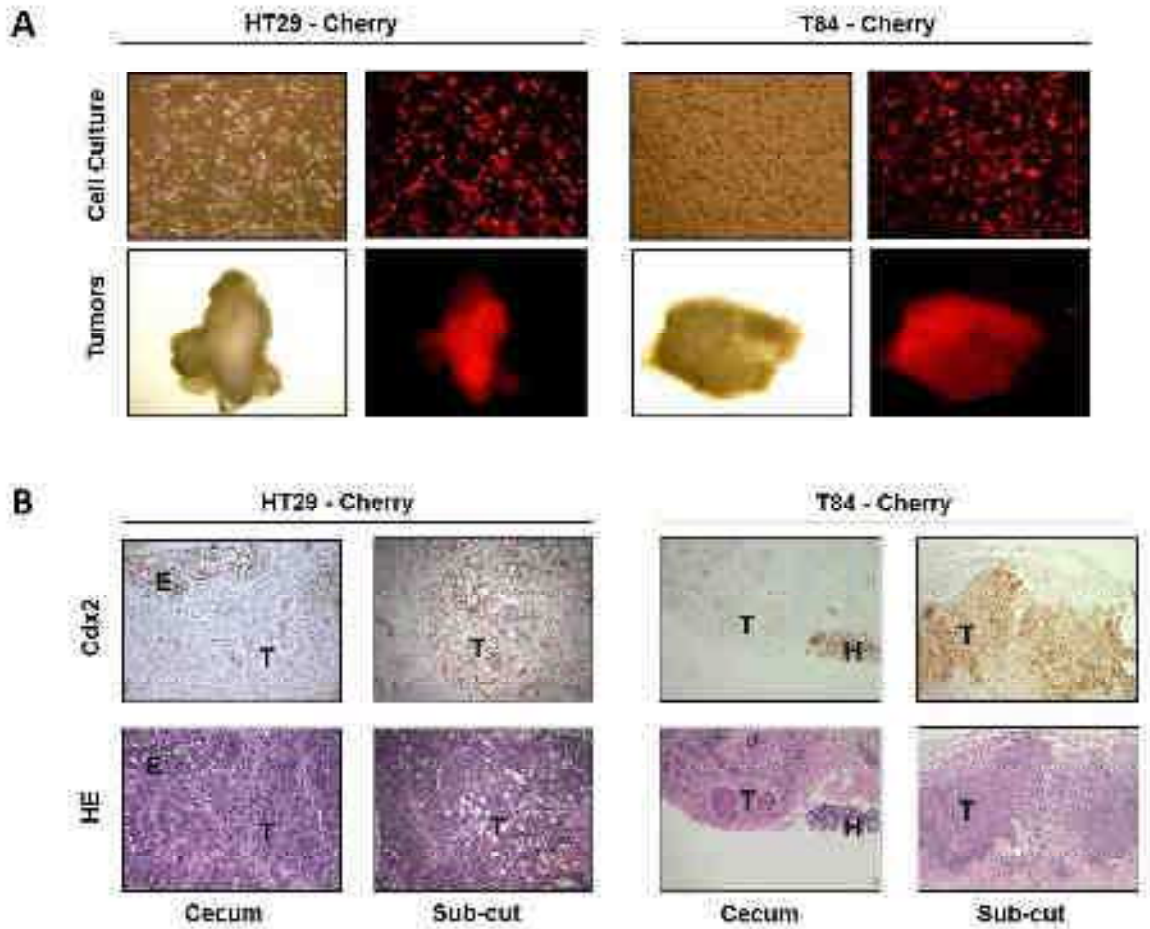


Figure 5: Validation of the T84-Cherry and HT29-Cherry cell lines

(A) The 3 selected clones of the HT29-Cherry and T84-Cherry cell lines present strong and homogeneous red fluorescence *in vitro* and in tumors formed 8 weeks after xenografting in nude mice. **(B)** Immunohistochemistry analysis of Cdx2 in the tumors arisen from xenografts under the skin (sub-cut) or into the cecum wall. HE: Hematoxylin-eosin staining; T: Tumor tissue; H: Host tissue (normal intestinal epithelium); E: Blood vessel.

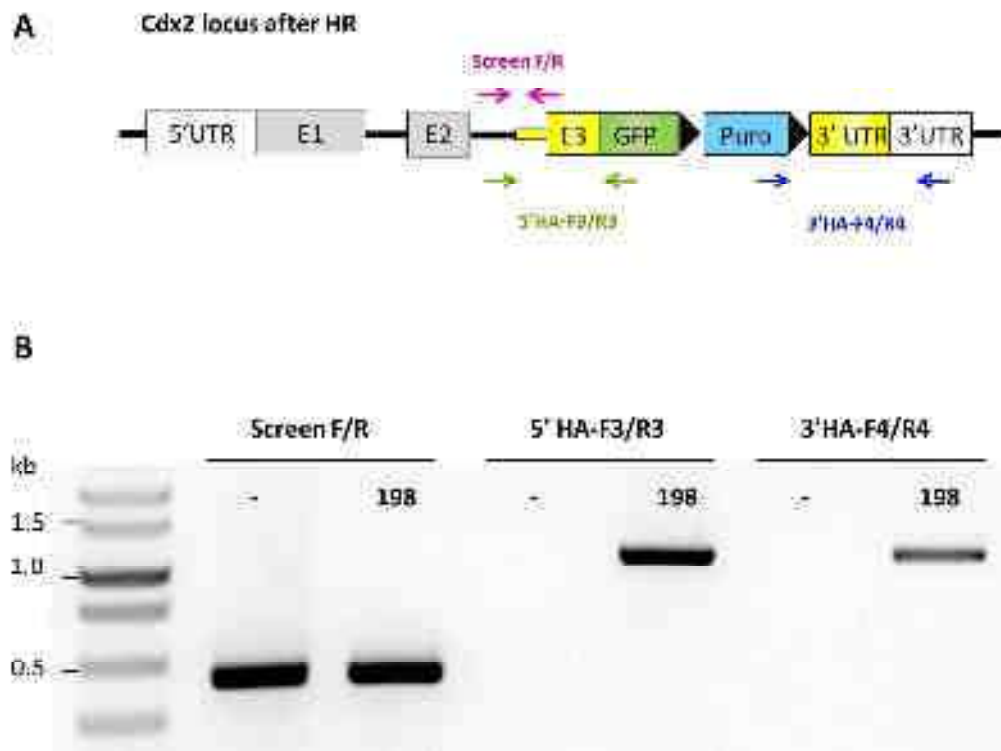


Figure 6: Detection of the somatic knock-in in clone 198

(A) The primer pairs 5'HA-R3/F3 and 3'HA-R4/F4 detect respectively integration of the knock-in cassette at the 5' extremity and the 3' extremity of the targeted locus. The Screen F/R primer detects a sequence found in wild-type HT29 cells and cells having integrated the knock-in cassette. **(B)** PCR on genomic DNA with primers located as shown in (A) on parental HT29 (-) and the clone having integrated the targeting cassette (198).

Discussion, Perspectives and Conclusion

1. Context

In invasive cells, Cdx2 expression is transiently lost, and re-expressed in metastasis, indicating that Cdx2 would make a good marker of tumor cell heterogeneity and plasticity. Therefore, I aimed at generating a Cdx2/GFP reporter cell line that permits to identify and characterize different colon cancer cell populations based on their level of Cdx2 expression in the tumors of nude mice. Interestingly, in two recent publications, a very similar approach was successfully used to study the heterogeneous transcriptional activity of β -catenin in colorectal tumors and its functional consequences. This was achieved by generating a reporter cell line for Wnt activity using the β -catenin reporter vector TOP-GFP (TOP: consensus binding sequences for TCF and activated β -catenin). After injection in nude mice, the β -catenin was indeed activated in a heterogeneous fashion in the tumors. Cells with different GFP fluorescence were sorted by FACS and analyzed afterwards on the functional and molecular level. In this way, interesting insights were obtained about the consequences of the heterogeneous activation of Wnt signaling for the stemness of cancer cells (Vermeulen et al., 2010; Horst et al., 2012). The dynamic contribution of the micro-environment (Ex: HGF secreted by myofibroblasts, local activation of the MAPK pathway) to the heterogeneity was also well highlighted. Therefore, these studies nicely confirm the adequacy of the type of experimental model I proposed to study tumor cell plasticity.

2. Discussion of the results obtained so far

In this context, I first created and validated the T84-Cherry and HT29-Cherry cell lines that stably express a red fluorescent protein to separate murine and human cells in xenografting experiments. Next, I generated a HT29-Cherry cell line that integrated the GFP coding sequence into the Cdx2 locus. Importantly, this cell line needs further validation to verify the expression of a Cdx2-GFP fusion protein in a pattern that reflects the expression of the wild-type Cdx2 gene.

To introduce the GFP coding sequence into the locus of Cdx2, a homologous recombination cassette was delivered by rAAVs into the cells. However, only 1 out of 675 screened clones was positively tested by PCR for correct recombination. This targeting efficiency is unexpectedly low (< 0.2%) compared to the targeting efficiencies reported in the literature (0.3%-13.9%) (Kohli et al., 2004). This can be due to several experimental circumstances. For instance, the virus stock that was used for transduction was not concentrated enough to generate a high number of

homologous targeting events. Indeed, the virus production could have been affected by the length of 4.9 kb of the targeting cassette, which actually corresponds to the maximal length for viral packaging (Kohli et al., 2004). This could have reduced the number of correctly packaged and functional virus particles. Another potential explanation for the low targeting efficiency might be that Cdx2 is not transcribed in HT29 cells and it has been reported that homologous recombination is more efficient in transcriptionally active loci (Nakai et al., 2003).

In general, the success of gene targeting depends on multiple factors and cannot be predicted a priori, as the efficiency of correct homologous recombination is extremely variable and depends on several factors that are sequence and cell type specific. In average, the targeting efficiency (defined as the proportion of correctly recombined clones) varies between 0.3% and 13,9 % (Kohli et al., 2004), but efficiencies up to 70% have been reported (Hirata et al., 2002). Remarkably, this variability can be observed for the same gene targeting construction delivered into different cell lines (Rogers et al., 2008). Thus, even if I was able to identify by PCR one positive clone, the somatic knock-in strategy can appear as a cumbersome and uncertain approach. Nevertheless, it represents the only option for cell lines if promoter fragments of the gene of interest are not able to recapitulate its regulation.

3. Continuation of the project

3.1 Validation of the recombinant cell line

The next steps will be to continue the validation of the positive cell line on the molecular level by confirming correct gene targeting through Southern blot and sequencing. Moreover, the expression of the Cdx2-GFP fusion protein will be verified through RT-qPCR and Western blot. Because HT29 cells do not express Cdx2 in cell culture, the cells will be treated with sodium butyrate, which induces Cdx2 expression *in vitro* (Domon-Dell et al., 2002). In addition, it will be necessary to check that the cells still form tumors with heterogeneous Cdx2 expression and that the GFP intensity correlates with Cdx2 levels.

3.2 Generation of the T84-Cherry reporter line

To complete the project, the T84-Cherry Cdx2 reporter cell line should also be generated. Cdx2 is expressed in this cell line, so the probability to integrate the targeting construction into the Cdx2 locus is increased. Moreover, the screening process for correct recombinants may be facilitated by the detection of the green fluorescence that should accompany constitutive Cdx2 expression in these cells.

3.3 Sorting and characterization of various cell populations

The Cdx2 reporter cell line will be injected into the cecum wall or under the skin of nude mice where they will proliferate and form tumors. The cells that exhibit homogenous expression of Cdx2 *in vitro* are expected to present heterogeneous Cdx2 expression *in vivo*. The cells of these tumors will be sorted by FACS into GFP/Cdx2 high and GFP/Cdx2 low cell populations. These different cell populations will be analyzed on the molecular level, by comparing their transcriptomes using DNA microarrays or RNA-Seq. This could lead to the identification of gene expression signatures associated to the high/low expression of Cdx2 in tumors. In addition, the functional properties of the different cell populations will be assessed. *In vitro*, their proliferative, migratory and invasive behavior will be determined. *In vivo*, their potential for tumor initiation (injection of limiting dilutions of cancer cells) will be tested and tumors will be analyzed on the histological and molecular level.

4. Potential outcomes

4.1 Confirm the implication of plasticity in the metastatic process

A correlation was established between the presence of disseminating cells in the stroma and the incidence of metastasis (Brabletz et al., 2005a; Prall, 2007). However, no formal proof has been provided that invading cells give rise to the metastasis forming cells, meaning that it would be possible that metastatic tumor cells did not transiently lose their epithelial phenotype and that only disseminated cells got mesenchymal features (Brabletz et al., 2005b). The use of the fluorescent Cdx2 reporter cell line may shed some light onto this process, as it will permit to follow the grafted cells and evaluate their ability to regain a heterogeneous Cdx2 expression pattern. Moreover, it will be possible to compare the clonogenic and tumor formation capacities of sorted GFP/Cdx2 low and GFP/Cdx2 high cells. If the cells that lost Cdx2 expression *in vivo* transiently acquired a more aggressive phenotype, this would confirm the importance of plasticity.

4.2 Identify new genes /pathways implicated in cancer cell plasticity

Molecular analysis of the reporter cells could help to identify new genes/pathways that are differentially regulated by the micro-environment and promote metastasis formation. These genes may constitute new targets in colorectal cancer therapy, but also molecular markers for diagnosis and prognosis. The identification and/or validation of diagnostic and prognostic markers for colorectal cancer cell progression may also be very important to make therapeutic

decisions. For instance, molecular markers may help to discriminate between stage II patients, the ones that would benefit from adjuvant chemotherapy and the ones with a low risk of recurrence.

4.3 Study of the regulation of Cdx2 target genes *in vivo*

Another possible use of the Cdx2 reporter cell lines is the study of the molecular consequences of Cdx2 expression gain/loss *in vivo*, which actually might be different from those observed *in vitro* because of the micro-environmental influence. In addition, it would be interesting to compare the functional consequences of different Cdx2 expression levels on target gene expression *in vivo*. In the Wnt pathway, different β -catenin concentrations can activate transcription of different sets of target genes and consequently the phenotype of the cells. For Cdx2, it has been shown that it is weakly expressed in proliferating cells and highly in differentiated ones (Verzi et al., 2010b). It would be interesting to determine if Cdx2 acts in a dose-dependent way on different sets of target genes and for this the Cdx2 reporter cell line would be an ideal tool.

4.4 Screening of drug libraries

Another interesting application of a Cdx2/GFP reporter cell line is the large scale screening of a library of chemical agents/small molecules in search of a compound that turns back on again Cdx2 expression in colon cancer cells. As Cdx2 expression inhibits metastasis formation in mice (Gross et al., 2008), its expression is associated to a good prognosis in human colon cancer and inversely correlates with metastasis formation (Choi et al., 2006; Bakaris et al., 2008), such a drug could be used in combination with currently prescribed cytotoxic agents in colorectal cancer therapy.

5. Conclusion

In summary, I have generated a cellular model (Cdx2/GFP reporter cell line), which after validation may be a valuable tool to study the molecular and functional consequences of tumor heterogeneity on tumor progression. This might in the long run lead to the discovery of better prognostic/diagnostic markers and/or the identification of new therapeutic targets to prevent or at least retard metastasis formation in colorectal cancer patients.

PROJECT 2:

Determination of the molecular relays of Cdx2

Cdx2 opposes elasticity of colon cancer cells through reorganization of the actin cytoskeleton and enhanced cell-cell contacts

Hinkel I.*, Platet N.*, Richert L., Senger B., Vautier D., Vannier M., Gaidon C., Voegel J.C., Freund J.N. & Gross I. The homeotic tumor suppressor Cdx2 opposes elasticity of colon cancer cells through reorganization of the actin cytoskeleton and enhanced cell-cell contacts. In preparation. * co-authors

“May every young scientist remember... and not fail to keep his eyes open for the possibility that an irritating failure of his apparatus to give consistent results may once or twice in a lifetime conceal an important discovery.”

Patrick Blackett
Experimental physicist – 1897 – 1974

Abstract

Carcinomas are the most frequent type of human malignancies and the vast majority of cancer deaths are caused by the formation of metastases rather than by the primary tumor itself. We have previously shown that Cdx2, a homeotic transcription factor essential for gut development, acts as a colon-specific tumor suppressor and opposes metastasis. Here, using a combination of biochemical, biophysical, and immunofluorescence techniques, we further investigated the molecular and cellular mechanisms promoted by Cdx2 that might antagonize tumor cell dissemination. Measurement of cell deformability by atomic force microscopy (AFM) showed that a monolayer of colon cancer cells expressing Cdx2 was significantly more rigid than the one formed by control cells. This increased stiffness correlated with enhanced adherens junctions and dramatic re-organization of the actin cytoskeleton into dense, well-aligned, stress fibers. We found that expression of Cdx2 in colon cancer cells stimulated the transcription of several Rho GEFs and activated Rho/ROCK/MLC and Rho/Dia1 signaling cascades able to induce the formation of stress fibers. Thus, down-regulation of Cdx2 during colon cancer progression may increase the elasticity of cancer cells and hence facilitate invasion and metastasis.

Introduction

Cancer is still a leading cause of death worldwide, with metastasis being the most fatal aspect of the disease and responsible for 90% of the lethality (Hanahan and Weinberg, 2000). Thus, from a clinical point of view, understanding the mechanisms involved in the metastatic process is a challenge of the utmost importance. The development of a tumor from a normal tissue is a complex multistage process, during which a benign precursor lesion progresses to a malignant tumor able to colonize secondary tissues (Hanahan and Weinberg, 2000). In carcinomas, metastasis involves primary tumor cells mobilization (detachment, invasion and migration through surrounding tissue matrix), intravasation into blood or lymphatic vessels, spreading to the capillary bed of distant organs, extravasation and growth of a secondary tumor (Gupta and Massagué, 2006). To achieve this highly selective processes, cancerous cells acquire new properties, change their proliferative, survival, adhesive, migratory and invasive abilities (Gupta and Massagué, 2006), but also exhibit new biomechanical and biophysical features (Suresh, 2007), through autonomous (irreversible genetic alteration of oncogenes or tumor suppressor genes) and non-autonomous (dictated by the tumor micro-environment) changes in expression and/or activity (Weinberg, 2008).

Cdx2 is one of the genes whose expression is decreased in the majority of colon cancer patients (Subtil et al., 2007), especially at the invasive front where cancer cells detach from the primary tumor, invade the surrounding stroma and disseminate into the rest of the body (Brabletz et al., 2004). Cdx2 encodes a homeotic transcription factor essential for various steps of embryonic development, including important aspects of intestinal identity and morphogenesis (Gao et al., 2009; Guo et al., 2004). In the adult, Cdx2 expression is restricted to the intestinal epithelium (Silberg et al., 2000), where it likely opposes cell proliferation and stimulates intestinal differentiation (Aoki et al., 2011; Lorentz, 1997; Suh and Traber, 1996), thus contributing to the homeostasis of the continuously renewing gut. Indeed, decrease of Cdx2 expression enhances colon cancer progression in two different mouse models, indicating that it acts as a tumor suppressor: i) treatment with the colon carcinogen AOM led to the apparition of invasive adenocarcinomas in the distal colon much earlier in Cdx2^{+/-} Knockout mice than in wild-type mice (Bonhomme et al., 2003); ii) Apc^{+/ Δ 716} Cdx2^{+/-} compound mutant mice exhibited much more polyps in the distal colon than Apc^{+/ Δ 716} mice (Aoki et al., 2003). In addition, forced

expression of Cdx2 decreased the incidence of metastases formed by colon cancer cells injected in nude mice (Gross et al., 2008), suggesting that the specific loss of Cdx2 expression at the invasion front contributes to tumor cell dissemination. Accordingly, inhibition of Cdx2 by siRNA silencing was shown to enhance *in vitro* migration of cancer cells (Gross et al., 2008).

In the present study, we have further explored the mechanisms by which Cdx2 opposes the invasion-metastasis process. We found that Cdx2 enhanced cellular junctions and activates a Rho signaling pathway, which upon formation of actomyosin stress fibers, increases the stiffness of colon cancer cells. The loss of Cdx2 in invasive cells is therefore likely to render them more deformable and hence give them the ability to migrate more easily through the surrounding stroma and capillaries.

Materials and Methods

Cell culture and inhibitor treatment

Human colon adenocarcinoma Caco-2/TC7 cells (Chantret et al., 1994) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 20% heat-inactivated Fetal Bovine Serum (FBS, Invitrogen), 1% essential amino-acids and antibiotics. Human colon adenocarcinoma SW480 cells (ATCC) were grown in DMEM supplemented with 10% FBS and antibiotics. HT29-Cdx2 inducible cells (clone TW6), generated from human colon adenocarcinoma HT29-16E/TR cells as described elsewhere (Gross et al., 2008), are stably modified to express Cdx2 upon doxycycline treatment. Cdx2 expressing TW6 cells and control TG8 cells were maintained in DMEM supplemented with 10% FBS, blasticidin 5 µg/ml and zeocin 100 µg/ml. Induction was performed by adding doxycycline (1 µg/ml, Sigma) to the culture medium.

Cell permeable Rho Inhibitor C3 transferase (CT04, Tebu-bio), ROCK inhibitor Y-27632 (Calbiochem) and MLCK inhibitor ML-7 (Sigma) were used at 5 µg/ml, 30 µM and 15 µM, respectively, and were added to the culture medium for 4 h.

Immunofluorescence staining and microscopy

Cells grown on Falcon glass culture slides were generally fixed for 15 min in 4% paraformaldehyde at room temperature. For mDia1 staining, cell fixation in ice-cold methanol for 10 min was used. Cells were then blocked and permeabilized in PBS containing 4% BSA and 0.3% Triton X100 for 1 h at room temperature, and then incubated sequentially in the same buffer with the primary antibody overnight at 4°C, and with the secondary antibody for 30 min at room temperature. TRITC-phalloidin (Sigma; 1/3000) staining was performed for 30 min at room temperature. Nuclei were stained with DAPI and slides were mounted with FluorSave Reagent from Calbiochem. Images were collected using the ApoTome technique (Zeiss) with the Axiovision software, or using confocal microscopy (Zeiss LSM 510 Meta) with the LSM Image Browser software.

Primary antibodies included: Cdx2 (CD88, Biogenex; 1/100), Tropomyosin (Sigma; 1/400), E-cadherin (Zymed; 1/200), Occludin (Zymed; 1/250), ZO-1 (Zymed, 1/30), mDia1 (BD Transduction Laboratories; 1/50) and Myosin Phosphatase (MYPT1, Covance; 1/1000). The secondary antibodies used were: goat anti-mouse, anti-rat or anti-rabbit, conjugated to Cy3 or

Alexa 488 fluorochromes (Jackson Laboratories; 1/1000).

Rho activation assay and immunoblotting

Rho activity was assessed with a Rho Activation Assay kit (Millipore): briefly, confluent cells grown in absence or presence of Doxycycline were serum-starved for 48 h before lysis with ice cold MLB buffer. 2.5 mg of cleared cellular extract were incubated with 35 μ l of agarose beads slurry coupled to GST-Rhotekin (\approx 25 μ g) for 45 min at 4°C with agitation. Beads were next washed 3 times with ice-cold MLB buffer and resuspended in Laemmli buffer. Pull-downs and inputs (raw cellular extracts, 25 μ g) were loaded on a 15% SDS-PAGE and analyzed by immunoblotting as described (Gross et al., 2005).

For all other analyses of protein content, cellular extracts were prepared as described (Gross et al., 2005), except that phosphatase inhibitor cocktails (I and II, Sigma) were added to the lysis buffer. The following primary antibodies were used overnight at 4°C: Cdx2 (Biogenex; 1/5000), Actin (Chemicon International; 1/15000), Rho (clone 55, Millipore; 1/250), phospho-Myosin light chain 2 (Ser19) and Myosin light chain 2 (Cell Signaling Technology; 1/1000).

Gene expression analysis

Total RNA was extracted from cells using Tri Reagent (Molecular Research Center) and 2 μ g were used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR reactions were performed in technical duplicates with diluted cDNAs (5x), Taqman Universal Master mix and gene-specific TaqMan probe and primers sets (TaqMan Gene Expression Assays, Applied Biosystems) on a 7500 real-time PCR System (Applied Biosystems). Analysis of the results was performed with the 7500 software v2.0.1 (Applied Biosystems) using the relative $\Delta\Delta$ Ct quantification method.

The following TaqMan Gene Expression Assays were used: Vav3 (Hs00916812_m1 specific of Vav3 v1 or NM_006113.4, Hs00919115_m1 specific of Vav3 v2 or NM_001079874.1, Hs00196125_m1 which recognizes both variants), MCF2 (Hs00930593_m1 which recognizes all variants), Cdx2 (Hs00230919_m1), TBP, Hs99999910_m1, Dia1 (Hs00946556_m1). Taqman Gene Expression Assays on the custom plate are presented in Table 1. TBP or GAPDH, 18S and TBP (custom plate) expression were used as endogenous controls to normalize all reactions.

Nanoindentation experiments

Nanoindentation experiments on cell monolayers were conducted at 37°C in CO₂-independent medium using the PicoForce microscope (Veeco) and PT.BORO.SN.5 cantilevers (0.02 N.m⁻¹ of spring stiffness, 5 µm of tip diameter, Novascan Technologies, Inc.). Displacement-controlled nanoindentation was carried out by loading at a rate of 1 µm.s⁻¹ up to a trigger force of 2 nN followed by unloading at the same rate. To derive the elastic modulus (E), the Hertzian relation was used, obtained for spherical tips between applied force (F) and resulting indentation (δ), namely $F = \frac{4}{3} \frac{E}{1-\nu^2} R^{1/2} \delta^{3/2}$ in which ν is the Poisson ratio and R is the tip radius. The Poisson ratio of the cell monolayer was taken to be 0.5, as is typical for soft biological materials (Touhami et al., 2003). Values of the elastic modulus were derived from the experimental force versus indentation curve over the indentation domain ranging from 20 nm up to 100 nm of indentation. A minimum of 20 µm interindentation spacing was considered to be sufficiently large for minimal interference with a neighboring residual inelastically deformed zone as well as residual stresses. For statistical analysis of nanoindentation experiments, histograms have been compared with a One-Way ANOVA test on ranks (i.e. Kruskal-Wallis test; Sigmastat, Systat Software), followed by pairwise comparison using Dunn's method.

Plasmids, siRNA and transfection

pFLAG-hCdx2 was obtained by first sub-cloning a RT-PCR fragment containing the human Cdx2 CDS into pCRII/TOPO (Invitrogen) and next inserting a HindIII-XbaI fragment from the resulting plasmid into pFLAG-CMV2 (Sigma). **pFLAG-hMiniCdx2** encoding a dominant negative isoform of Cdx2 has been described (Renouf et al., 2012).

pFLAG-hCdx2 R189A and **pFLAG-hCdx2 Q234K** mutants of Cdx2 were generated with the Quikchange II XL Site-Directed Mutagenesis Kit (Stratagene) using pFLAG-hCdx2 as template and the following mutagenesis primers:

pFLAG-hCdx2-R189A

(F: AAAGACAAATATGCAGTGGTGTACACGGACC; R: GGTCCTGTACACCACTGCATATTTGTCTTT)

pFLAG-hCdx2-Q234K

(F: GGTAAAATCTGGTTAAGAACCGCAGAGC; R: GCTCTGCGTTCTTAAACCAGATTTAACC).

To obtain **pVav3 v2-pGL3** and **pVav3 v1-pGL3** constructs, DNA fragments of different sizes were generated by PCR using HT29 cells genomic DNA, the PCRx Enhancer System (Invitrogen) and primers specific for the promoter region of the small (v2) or long (v1) isoform, including the sequence for the 15 bp extensions complementary to the vector ends. These primers were for the small isoform v2 (2.7 kb fragment F: TTTCTCTATCGATAGGTACCTTAGTGTCAGTGAA CAGGCTATTGC; 0.5 kb fragment F: TTTCTCTATCGATAGGTACCTTAAGTTCCATCTTTAACTCAAAGT; both fragments R: GATCGCAGATCTCGAGACCTTAGCTTTCTCTGCAGAGTGG) and for the long isoform v1 (1.8 kb fragment F: TTTCTCTATCGATAGGTACCTCAGCGCTGAGTGAAACAGTG; R: GATCGCAGATCTCGAGCCGACGGCTCCGGGACGC). The extensions of the PCR products complementary to the vector ends permitted insertion with the “In Fusion HD” cloning enzyme (Clontech) into pGL3-basic (Promega), previously linearized (KpnI-XhoI) and gel-purified. pRL-null was also from Promega.

Stealth/siRNA duplex oligoribonucleotides were from Invitrogen: CDX2HSS141546 (siRNA@Cdx2 546) and low GC Duplex #2 as negative control.

Plasmid transfections were performed with Jetprime (Polyplus Transfection) or JetPEI (Polyplus Transfection, Luciferase assays) as recommended. siRNA duplexes (1 nM final) were transfected with Lipofectamine RNAiMAX (Invitrogen).

Chromatin immunoprecipitation

Fixation of cells was performed with 1% (v/v) formaldehyde for 10 min at room temperature and quenched with 0.125 M Glycine for 5 min. ChIP experiments were carried out using the EZ-Magna ChIP™ G Chromatin Immunoprecipitation kit as recommended by the supplier (Millipore). Sheared crosslinked chromatin from $\sim 10^6$ cells was incubated overnight at 4°C with 1 µg of mouse IgG (Upstate Cell Signaling) or with mouse anti-Cdx2 (Biogenex) or anti-Flag (Sigma) antibodies. Input corresponds to non-immunoprecipitated, sheared and crosslinked chromatin from $\sim 10^5$ cells (1%). PCR analysis was performed with Red'y'Star Mix (Eurogentec), 1/25th of immunoprecipitated DNA as template and primers located in the human Vav3 v2 (short isoform) upstream region (F: TCA TGG AAC ATG GTG TGA CAA CTA CTG A; R: CTA CTG GTG AGT TAG AGC CTG AAC T; annealing: 63°C; product: 335 bp) or Vav3 v1 (long isoform) upstream region (F: TCACTAAGGATCCCAGAACAGC; R: TCAGCCTATTCCTCCTAGTTGC; annealing 57°C; final MgCl₂

concentration 2,5 mM; product: 339 bp).

Luciferase assays

Luciferase assays were performed with the Dual-Luciferase Reporter Assay (Promega) as described (Gross et al., 2008). Data presented correspond to a representative experiment performed in triplicate.

Results

1. Cdx2 enhances the stiffness of a HT29 colon cancer cell monolayer

We previously showed that forced expression of Cdx2 via Doxycyclin in HT29 colon cancer cells decreased the ability of these cells to form metastases in nude mice (Gross et al., 2008). Given that successful metastasis involves profound modifications of the morphology and function of tumoral cells, we wondered if restoration of Cdx2 expression would impact on their biomechanical properties. Thus, we compared control and Cdx2-expressing HT29 monolayers (**Figure 1A**) using atomic force microscopy (AFM). Thickness of the monolayers (measured by confocal imaging and cytoplasmic dye) was found to be equivalent in both cases (18-25 μm , data not shown). Likewise, roughness of the monolayers (evaluated by topographic imaging with AFM in tapping mode) was similar at the scale of the probe/cell monolayer contact (data not shown). In contrast, elasticity examined by colloidal nanoindentation was modified in the presence of Cdx2: indeed, data collected in the approach and retraction of the tip were different (**Figure 1B**) and analysis of approach curves using the Hertz model (**Figure 1C**) indicated a significant hardening of Cdx2-expressing monolayers (average elastic modulus: 404 \pm 36 Pa) compared to control monolayers (average elastic modulus: 278 \pm 30 Pa). Thus, Cdx2 expression can reduce the elasticity of a colon cancer cell monolayer.

2. Cdx2 expression correlates with enhanced stress fiber assembly

Stiffness of a cell monolayer depends on various factors, namely the strength of cellular adhesion and the tension of the cellular cytoskeleton. We first examined the Actin microfilament network which plays a major role in cellular elasticity. Confocal immunofluorescence showed the presence of two types of Actin distribution in confluent HT29 monolayers: radial Actin bundles connecting the perinuclear zone to the cell surface and ventral Actin bundles running parallel underneath the plasma membrane. The radial Actin filaments were not affected by Cdx2 expression in HT29 cells (data not shown). In contrast, organization of the ventral Actin filaments in robust stress fibers was clearly enhanced in Cdx2-expressing HT29 monolayers: stress fibers were generally thicker and bundles appeared more parallel to each other than in control HT29 monolayers (**Figure 2A**). Similar results were obtained with the distribution of the Actin-binding protein Tropomyosin (**Figure 2B**). In addition, we examined by Western blot the phosphorylation

of Myosin Light Chain (MLC) at Serine 19 in extracts of confluent control or Cdx2-expressing HT29 cells. As shown on **Figure 2C**, while the amounts of MLC were unchanged by Cdx2 expression, its phosphorylation was enhanced, indicating greater Myosin activation and Actin binding (Totsukawa et al., 2000) than in control cells. Altogether, these results show a positive correlation between Cdx2 expression and the assembly of robust, well-organized, stress fibers.

3. Cdx2 can stimulate Rho GTPase activity

Actin stress fiber formation depends on signaling by members of the Rho family of small GTPases, especially RhoA (Pellegrin and Mellor, 2007). Transcript and protein levels of RhoA were not affected by Cdx2 expression in HT29 cells (**Supplementary Table 1** and **Figure 3A**). In contrast, Rho activity, assessed by binding of the active GTP-bound form to Rhotekin, was increased in HT29 cells expressing Cdx2 compared to control cells (**Figure 3A**). In addition, we examined the impact of Cdx2 on stress fiber challenged by Rho inhibition. Treatment of Cdx2-expressing cells with CT04, a cell-permeable version of the C3 Rho inhibitor, destroyed the stress fibers but to a weaker extent than in the control cells (**Figure 3B**). Importantly, expression of Cdx2 for 48h after a CT04 treatment promoted the formation of new stress fibers (data not shown). Thus, Cdx2 can activate Rho signaling and favors stress fibers recovery/stability upon Rho inhibition.

4. Cdx2 can affect several effectors of Rho-induced stress fiber formation

Formation of thick, parallel, stress fibers upon Rho activation requires both increased Actomyosin contractibility and Actin nucleation (Pellegrin and Mellor, 2007). Actomyosin contractibility depends on the phosphorylation of MLC at Ser19, which is achieved by different signaling pathways depending on the cell type considered. Several of these pathways involve the activation of the Ser/Thr kinase ROCK by RhoA. ROCK can either directly phosphorylate MLC or indirectly increase its phosphorylation by activating another kinase (ZIPK, in smooth muscle cells) or by inhibiting the MLC phosphatase. The MLCK can also directly phosphorylate MLC (Pellegrin and Mellor, 2007). To gain further insight into the molecular pathway leading to MLC phosphorylation upon Cdx2 expression in HT29 cells, we examined the involvement of several effectors using specific inhibitors. We first checked the impact of Cdx2 expression on stress fiber recovery or stability upon treatment with the ROCK inhibitor Y27632 (**Figure 4** and data not

shown). In both cases, slightly more stress fibers were observed in the presence of Cdx2, but it was less striking that was observed upon Rho inhibition (**Figure 3B**). We also used the ML7 inhibitor of MLCK but achieved only minimal stress fibers destruction that was not affected by Cdx2 expression (data not shown). Similarly, Cdx2 had no impact on the localization (i.e. activation) of the MLC phosphatase (data not shown). Thus, it is likely that phosphorylation of MLC upon Cdx2 expression is achieved directly by ROCK, following RhoA activation.

We next checked if other important effectors of Rho were sensitive to Cdx2 expression. Among these, the formin Dia1, which nucleates the polymerization of Actin filaments from cell membranes, is critical for the formation of parallel, well-aligned stress fibers (Pellegrin and Mellor, 2007). Dia1 transcripts assessed by RT-PCR were barely increased by Cdx2 expression (data not shown). In contrast, sub-cellular localization of Dia1 proteins examined by immunofluorescence was modified: in control cells, Dia1 staining appeared mostly diffuse in the cytoplasm, whereas in Cdx2-expressing cells it was re-enforced at the membrane, indicating its increased activity (**Figure 5**, top panels). Altogether, these results are consistent with Rho activation by Cdx2 translating in both increased Actomyosin contractibility via ROCK and Actin nucleation via Dia1, and leading to the formation of thick, well-organized stress fibers.

5. Cdx2 re-enforces adherens junctions

Actin polymerization has been shown to provide the driving force for the formation of cell-cell junctions, which contribute to tissue rigidity. Thus, we examined by immunofluorescence components of cell-cell junctions in monolayers of HT29 cells expressing or not Cdx2. Tight junction components ZO1 and Occludin were unaffected by Cdx2 (data not shown). In contrast, the adherens junction component E-cadherin was clearly modified: membrane staining of E-cadherin was discontinuous in control cells, whereas it appeared denser and more regular in Cdx2-expressing cells (**Figure 5**, middle panels). Importantly, the E-cadherin staining co-localized with Dia1 staining at the membrane (**Figure 5**, bottom panels), consistent with the proposed role of this Actin nucleation factor in controlling cadherin-mediated junctions (Carramusa et al., 2007).

6. Cdx2 can regulate the transcription of several Rho GEFs

To clarify how Cdx2 enhances Rho activity, we hypothesized that Cdx2 might stimulate the transcription of a Rho regulator, for instance a Rho GEF, which exchanges GDP for GTP to generate the activated Rho form. Therefore, RNA were extracted from HT29 cells expressing or not Cdx2 and analyzed by RT-qPCR with a custom-made microplate containing 28 Rho GEF TaqMan probes to assess their expression at once. Of note, the Rho GEFs were selected on their ability to stimulate RhoA activity since RhoA plays a critical role in stress fibers formation (Pellegrin and Mellor, 2007). Strikingly, only 2 of these Rho GEFs exhibited enhanced levels of transcripts upon Cdx2 expression (**Figure 6A** and **Supplementary Table 1**): Vav3 expression was increased up to 5-fold, whereas MCF2/Dbl showed a more modest increase of nearly 2-fold. Time-course experiments showed enhanced expression of both genes already after 2 days of Cdx2 expression, with Vav3 stimulation always stronger than that of MCF2/Dbl (**Figure 6B, C**), leading us to further investigate a direct regulation of Vav3 by Cdx2.

A long (5.4 kb: v1) and a short (3.4 kb: v2) transcript have been described for Vav3. Thus, we checked by RT-qPCR the impact of Cdx2 levels on both variants using specific TaqMan probes. Both variants had their expression stimulated by Cdx2, but the transcripts corresponding to the shortest isoform of Vav3 were prominent in HT29 cells and their level was enhanced 5-times upon Cdx2 expression, suggesting that they account for most of the stimulation previously observed with the non-discriminating Taqman probe (**Figure 6D**). Similar results were obtained in the human colon cancer cell line SW480 (data not show). In contrast to this, silencing of Cdx2 expression by RNA interference in the human colon cancer cell line Caco2/TC7, which expresses endogenously the short isoform Vav3 v2 but not the long one Vav3 v1, led to a slight but significant decrease of Vav3 v2 expression (**Figure 6E**). Of note, the partial silencing of Cdx2 in these cells led to a similar decrease in the expression of its target gene Cdh17 (**Figure 6E**).

We next examined the 5' regions of the long and the short Vav3 isoforms and found several potential Cdx2 binding sites. To determine if Cdx2 was able to bind to the Vav3 v2 or v1 upstream region, we performed chromatin immunoprecipitation (ChIP) experiments in HT29 and SW480 cells: upon overexpression of Cdx2, a DNA fragment corresponding to the Vav3 isoforms v2 or v1 upstream region was amplified by PCR in Cdx2 immunoprecipitates (data not shown and

Figure 7A). Therefore, we sub-cloned 2.7 kb and 0.5 kb 5' regions of the short Vav3 isoform upstream of a Luciferase reporter gene to test their sensitivity to Cdx2 overexpression. Upon transfection of Cdx2 in HT29 or SW480 cells, expression of both reporter constructs was increased in the presence of Cdx2 (**Figure 7B, C**). In SW480 cells, mutant Cdx2 proteins unable to bind DNA (point mutations in the homeodomain (Aoki et al., 2011)) did not stimulate the Vav3 v2 reporter gene (**Figure 7C**). In addition, miniCdx2, a dominant negative form of Cdx2 without the transactivation domain (Renouf et al., 2012; Modica et al., 2009), was not able to stimulate the Vav3 v2 reporter and even decreased the stimulation by wild-type Cdx2, suggesting competition for the binding sites (**Figure 7D**). Similar results were obtained with a 2.3 kb 5' region of the long Vav3 isoform that was cloned upstream of a Luciferase reporter gene (data not shown). Altogether, these results suggest that Cdx2 directly regulates the transcription of Vav3.

Figures

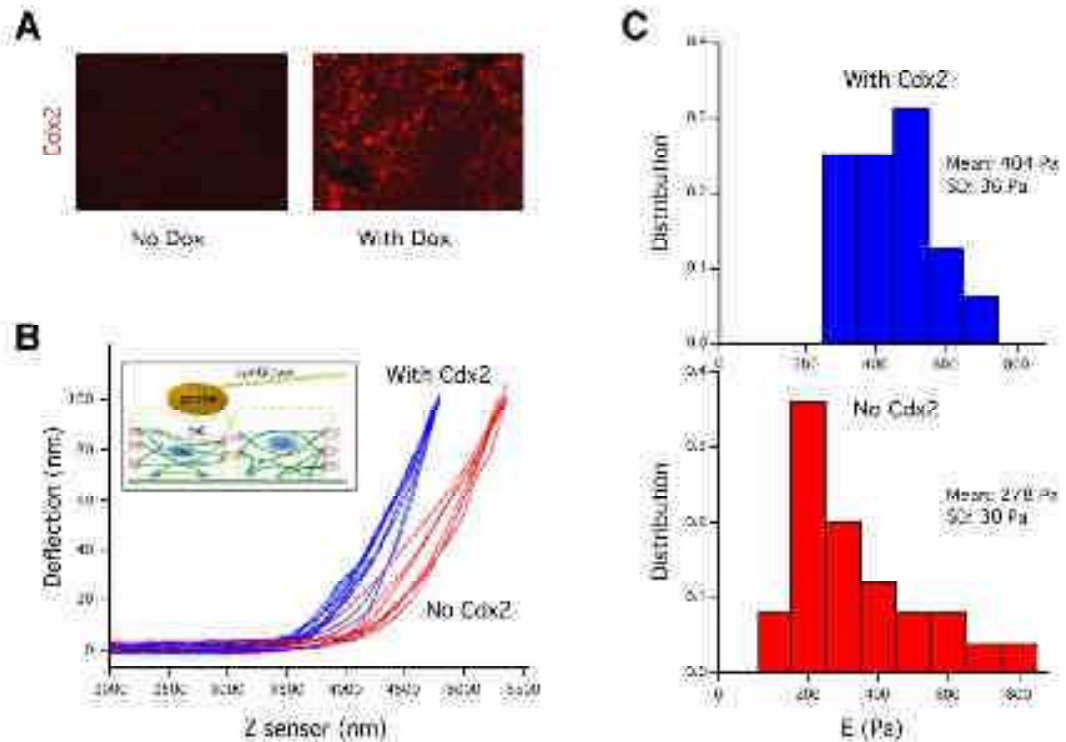


Figure 1: Cdx2 enhances the stiffness of a HT29 colon cancer cell monolayer

HT29-Cdx2 cells were seeded on coverslips and treated or not for 8-11 days with Doxycycline to induce Cdx2 expression. **(A)** Immunofluorescence analysis of cells with anti-Cdx2 antibody (original magnification: 200X). **(B)** Raw indentation curves obtained by atomic force microscopy with sphere probe on HT29 cell monolayers without (red lines) and with (blue lines) Cdx2 expression. The plot shows the deflection of the cantilever versus the vertical position of the sensor Z. In inset, a schematic representation of the experience. **(C)** Representative cell stiffness distributions for HT29 cell monolayers without (red bars) and with (blue bars) Cdx2 expression obtained after computation of the elastic modulus from the nanoindentation data shown in (B).

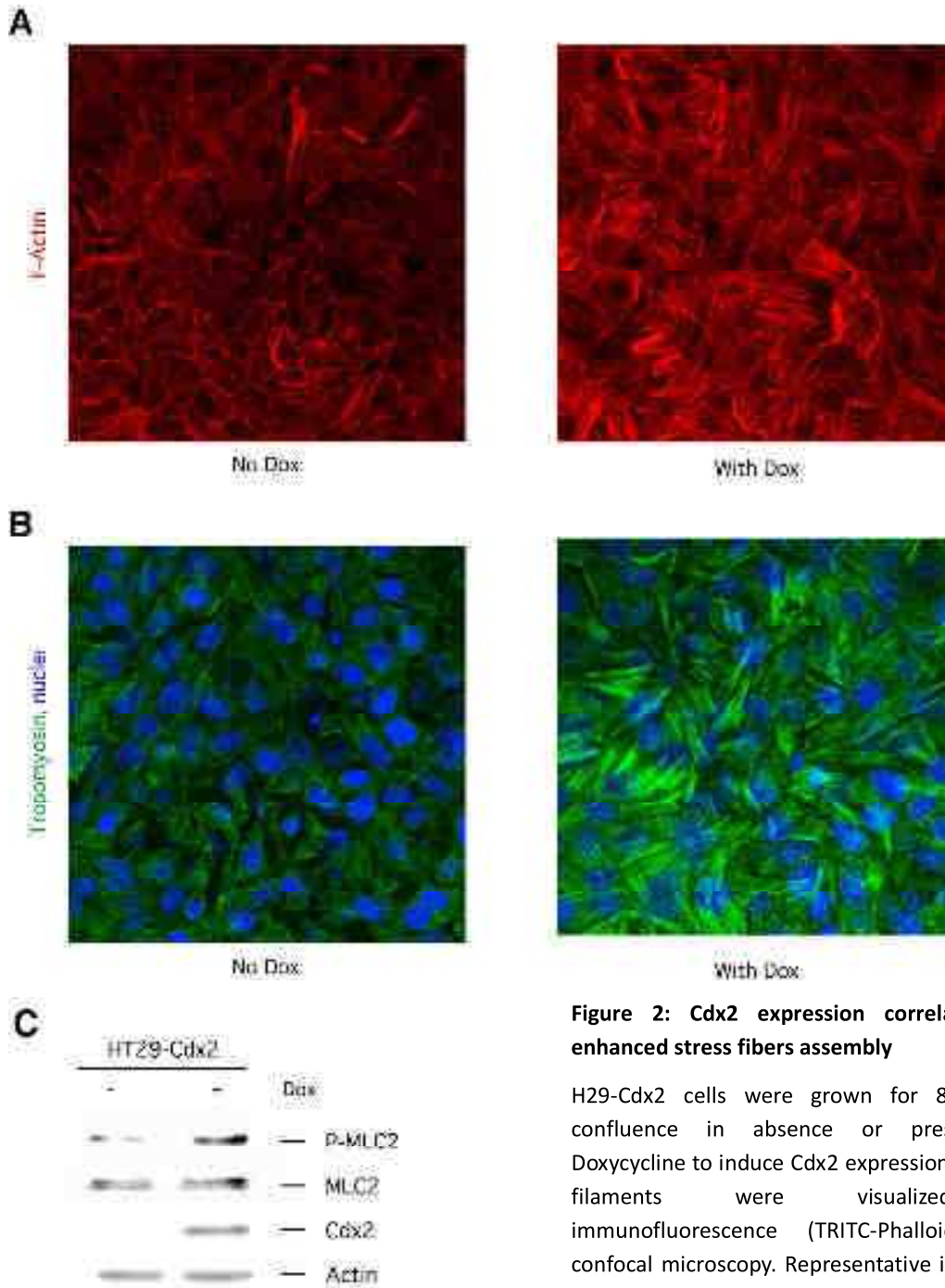


Figure 2: Cdx2 expression correlates with enhanced stress fibers assembly

H29-Cdx2 cells were grown for 8 days to confluence in absence or presence of Doxycycline to induce Cdx2 expression. **(A)** Actin filaments were visualized by immunofluorescence (TRITC-Phalloidin) and confocal microscopy. Representative images are shown (original magnification: 400X). **(B)** As in (A) with detection of Tropomyosin and nuclei (DAPI). **(C)** Lysed proteins were analyzed by Western blotting using the indicated antibodies (P-MLC2: Ser19-phosphorylated MLC2; MLC2: total MLC2).

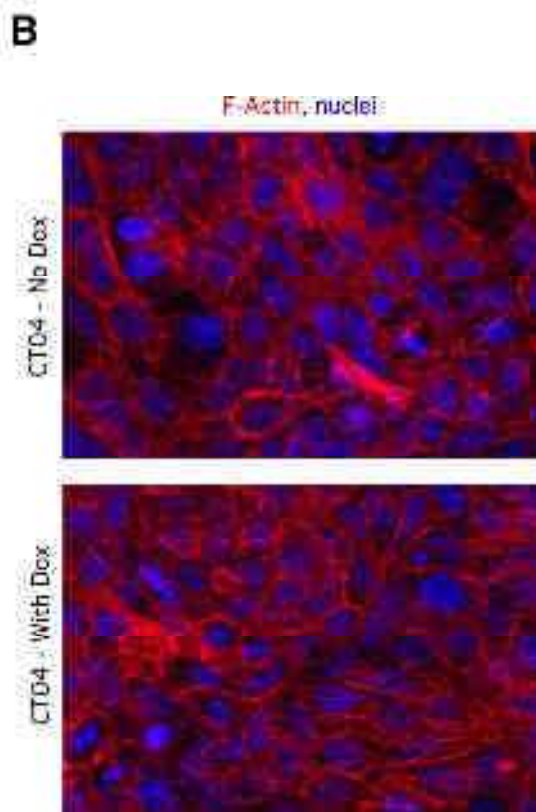
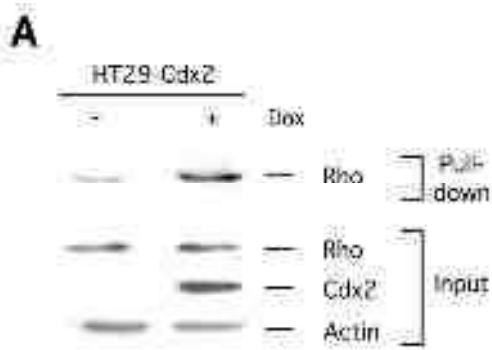


Figure 3: Cdx2 can stimulate Rho activity

(A) H29-Cdx2 cells were grown to confluence in absence or presence of Doxycycline to induce Cdx2 expression. Active Rho in cellular extracts was detected by Western blot after pull-down by GST-Rhotekin beads. Inputs (1%) were also analyzed by Western blot with the indicated antibodies. **(B)** H29-Cdx2 cells were grown to confluence on glass coverslips in absence or presence of Doxycycline to induce Cdx2 expression, and treated with the Rho inhibitor CT04 for 4h. After fixation, cells were stained with TRITC-Phalloïdin to detect F-Actin (red) and DAPI to detect nuclei (blue). Representative images are shown (original magnification: 400X).

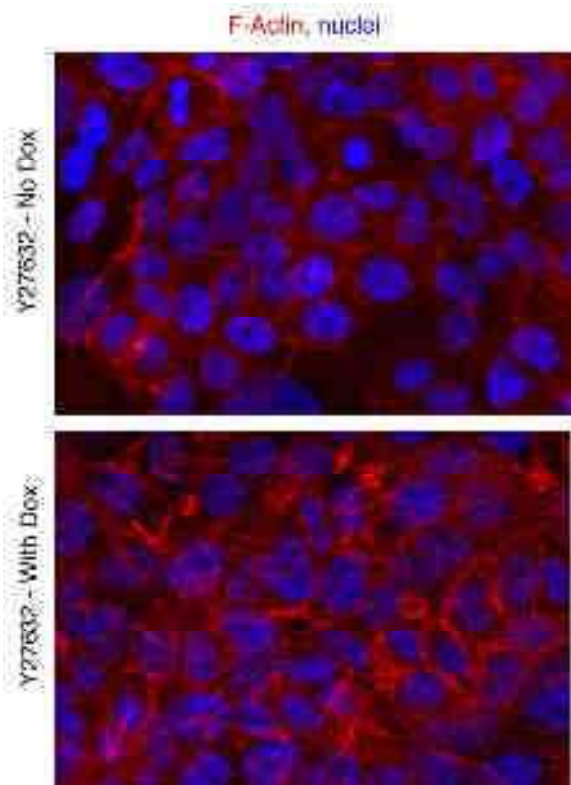


Figure 4: Cdx2 expression enhances stress fibers recovery after ROCK inhibition

H29-Cdx2 cells were grown to confluence on glass coverslips, treated with the ROCK inhibitor Y27632 for 4h and left to recover in absence or presence of doxycycline for 48h. After fixation, cells were stained with TRITC-Phalloïdin to detect F-Actin (red) and DAPI to detect nuclei (blue). Representative images are shown (original magnification: 400X).

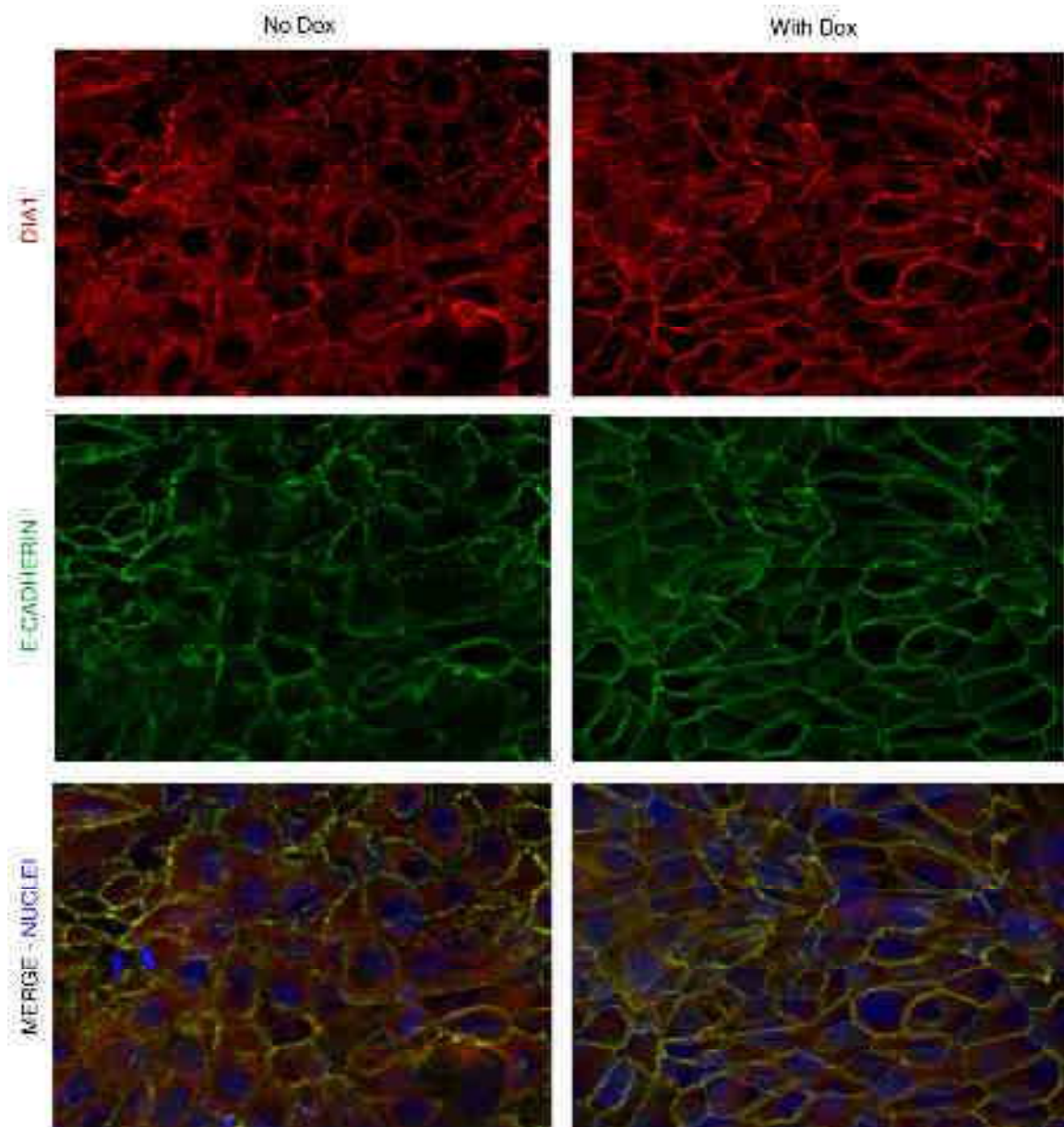


Figure 5: Cdx2 re-enforces adherens junctions

HT29-Cdx2 cells were grown to confluence in the absence or presence of Doxycycline and analyzed by immunofluorescence. The top panels show Dia1 staining (red), the middle panels show E-cadherin staining (green) and the bottom panels correspond to the merged fluorescence (yellow) of the Dia1 and E-cadherin stainings. Original magnification 400X.

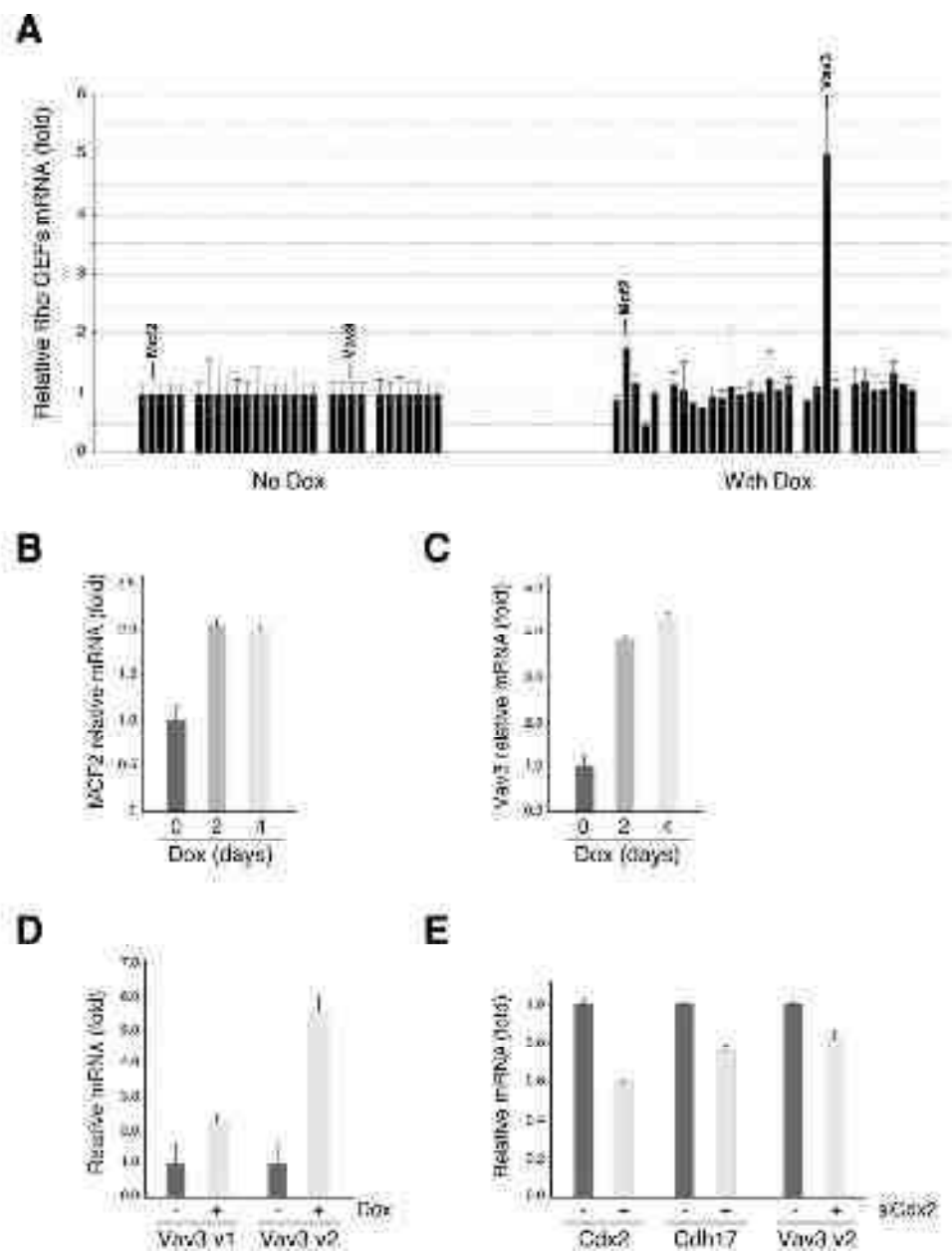


Figure 6: Cdx2 induces the expression of several Rho GEFs

(A) HT29-Cdx2 cells were grown in the absence or presence of Doxycycline (Dox) for 7 days before RNA extraction. mRNA levels for 28 RhoA GEFs, RhoA and 3 control genes were analyzed by RT-qPCR. A representative experiment performed in triplicates is shown, with the level of each gene in the absence of Dox being set at 1 (\pm SD). **(B), (C)** As in (A) with Dox treatments of 2 or 4 days and mRNA levels of MCF2/Dbl or Vav3 (all transcripts) respectively. **(D)** As in (A) but levels of the long (v1) and short (v2) transcripts of Vav3 were examined. **(E)** Caco2/TC7 cells were transfected for 3 days with control (-) or Cdx2 (+) siRNA before RNA extraction and RT-qPCR analysis of the indicated transcripts. For each gene, the level of expression (\pm SD, normalized with TBP) upon transfection of control siRNA was set at 1.

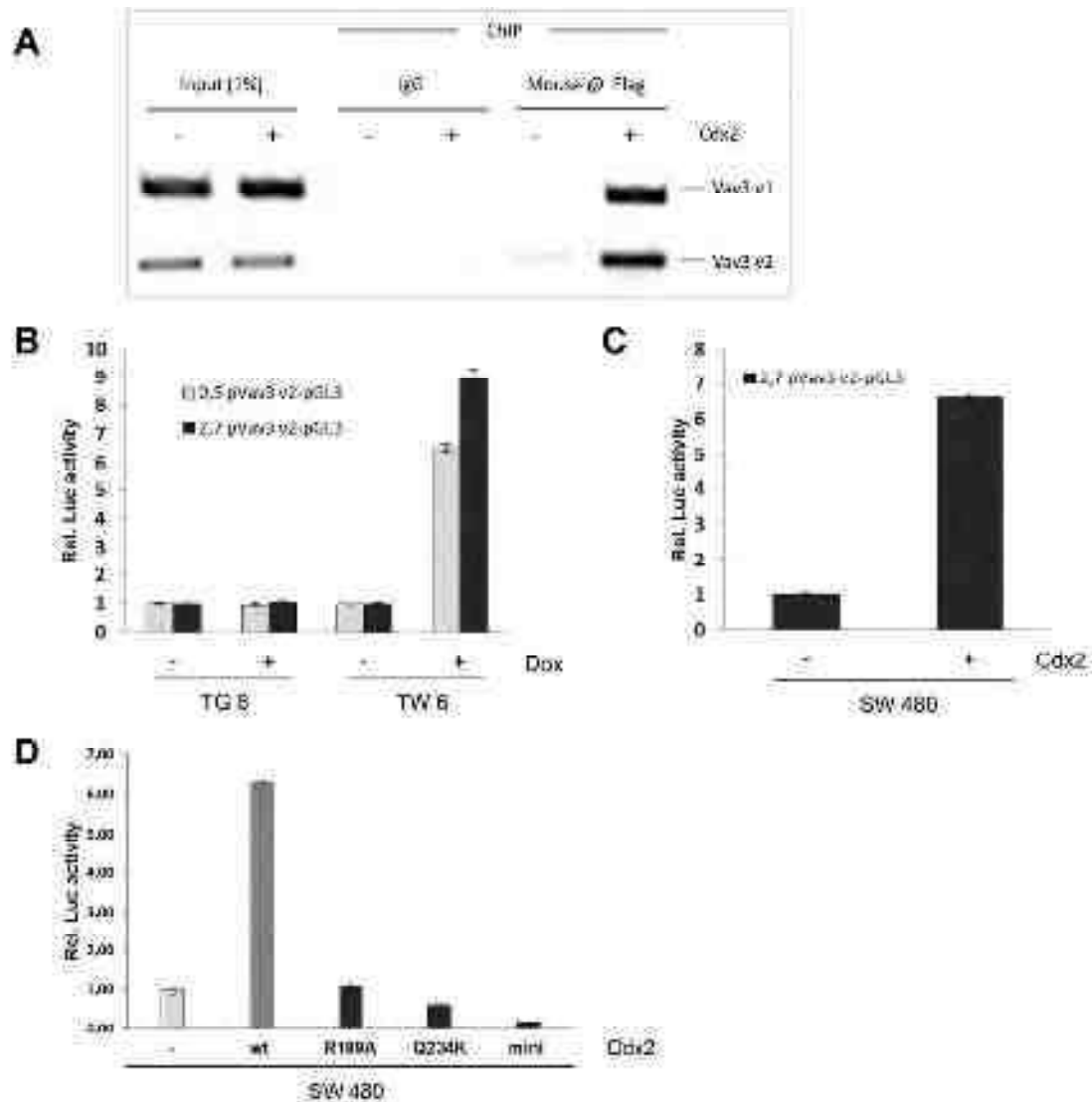


Figure 7: Cdx2 stimulates the transcription of Vav3 v2 and v1

(A) Chromatin of SW480 cells transfected with Flag-tagged Cdx2 (+) or not (-) was subjected to immunoprecipitation (CHIP) with an anti-Flag antibody or control IgG before PCR detection of a Vav3 v1 or Vav3 v2 promoter fragment. PCR was also performed with 1% of the bulk DNA (input) to ensure equivalence of the samples. **(B)** HT29 (TW6 expressing Cdx2 or TG8 negative control expressing GFP upon doxycycline induction) cells were treated or not with doxycycline and transfected with the indicated Vav3 v2-pGL3 reporter plasmid, as well as a promoter-less renilla reporter plasmid (internal control), before lysis and luminescence measurement. Relative promoter activity (\pm SD, normalized with renilla values) of each of the Vav3 v2-pGL3 reporter without doxycycline was set at 1. **(C)** as in B with SW480 cells transfected with empty plasmid (-) or expressing Cdx2 (+) and the 2.7 pVav3v2-pGL3. **(D)** As in B with SW480 cells transfected with 0.5 pVav3v2-pGL3 and an empty vector (-), wild type Cdx2 (wt) or mutated Cdx2 (R189A, Q234K, mini).

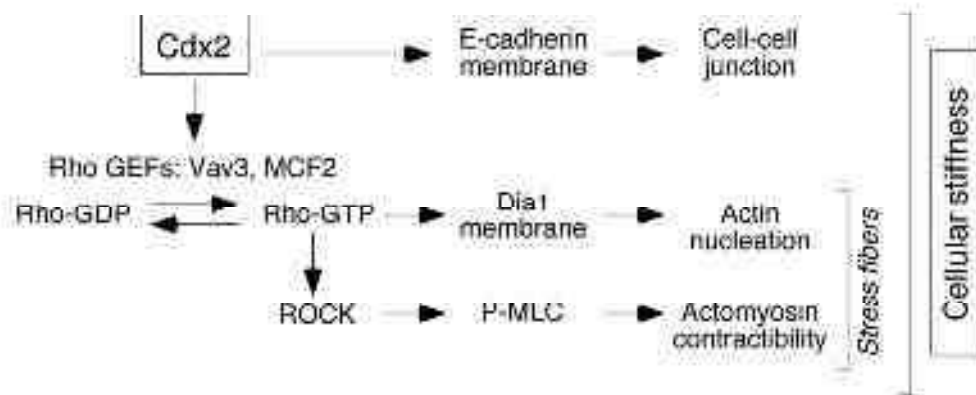


Figure 8: Molecular relays of Cdx2 involved in enhanced cellular stiffness

Cell-cell junctions are increased through enhanced membrane localization of E-cadherin. Cdx2 also stimulates the transcription of several Rho GEFs, enhances Rho activity, leading to a reorganization of the Actin cytoskeleton.

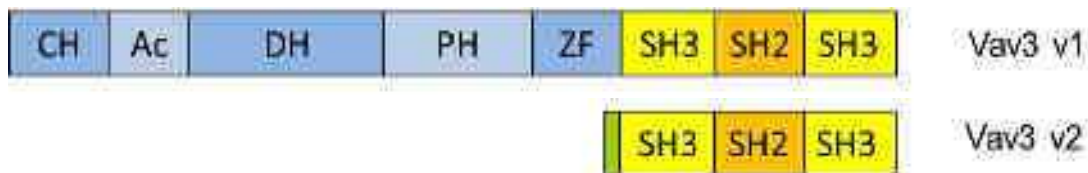


Figure 9: Vav3 isoforms

The long Vav3 isoform v1 exhibits a calponin-homology domain (CH), an acidic motif (Ac), a DBL-homology domain (DH) that binds to the RhoGTPase, a pleckstrin-homology domain (PH), a zinc-finger domain (ZF) and a SRC-homology 2 domain (SH2) flanked by 2 SRC-homology 3 domains (SH3). The short Vav3 isoform v2 only contains a specific sequence of 8 amino acids and the SH2-SH3-SH2 domains.

Supplementary information

| Sample | Gene - TaqMan Probe | RQ | RQ Min | RQ Max | Ct Mean | ΔCt Mean | ΔCt SD |
|----------|-------------------------|------|--------|--------|---------|------------------|----------------|
| No Dox | 18S-Hs00999901_s1 | | | | 5.78 | | |
| No Dox | ABR-Hs00241607_m1 | 1.00 | 0.85 | 1.17 | 25.97 | 7.07 | 0.23 |
| No Dox | AKAP13-Hs00180747_m1 | 1.00 | 0.94 | 1.18 | 22.87 | 5.03 | 0.25 |
| No Dox | ARHGAP14-Hs00180327_m1 | 1.00 | 0.96 | 1.17 | 24.92 | 5.08 | 0.23 |
| No Dox | ARHGAP10L-Hs00737885_m1 | 1.00 | 0.73 | 1.08 | 35.63 | 16.40 | 0.48 |
| No Dox | ARHGAP11-Hs00267600_m1 | 1.00 | 0.94 | 1.06 | 24.26 | 5.43 | 0.24 |
| No Dox | ARHGAP12-Hs00229661_m1 | 1.00 | 0.86 | 1.17 | 23.97 | 5.07 | 0.22 |
| No Dox | ARHGAP15-Hs00299097_m1 | 1.00 | 0.65 | 1.17 | 36.83 | 16.78 | 0.29 |
| No Dox | ARHGAP17-Hs00237825_m1 | 1.00 | 0.73 | 1.07 | 26.44 | 7.60 | 0.48 |
| No Dox | ARHGAP18-Hs00248726_m1 | 1.00 | 0.85 | 1.17 | 25.88 | 7.05 | 0.23 |
| No Dox | ARHGAP24-Hs00190884_m1 | 1.00 | 0.94 | 1.18 | 26.88 | 9.06 | 0.25 |
| No Dox | ARHGAP24-Hs00218804_m1 | 1.00 | 0.84 | 1.18 | 27.22 | 8.98 | 0.26 |
| No Dox | ARHGAP4-Hs00209707_m1 | 1.00 | 0.88 | 1.17 | 30.82 | 11.98 | 0.23 |
| No Dox | ARHGAP5-Hs00180553_m1 | 1.00 | 0.86 | 1.17 | 26.80 | 8.07 | 0.22 |
| No Dox | BCR-Hs00244731_m1 | 1.00 | 0.76 | 1.08 | 26.25 | 7.42 | 0.35 |
| No Dox | EC22-Hs00216456_m1 | 1.00 | 0.88 | 1.17 | 23.86 | 5.02 | 0.23 |
| No Dox | FARP1-Hs00195010_m1 | 1.00 | 0.84 | 1.18 | 23.22 | 4.90 | 0.28 |
| No Dox | FLJ10357-Hs00218283_m1 | 1.00 | 0.81 | 1.24 | 25.54 | 7.10 | 0.31 |
| No Dox | GAPDH-Hs00999905_m1 | | | | 16.74 | | |
| No Dox | GEF1-Hs00611102_m1 | 1.00 | 0.86 | 1.17 | 30.82 | 11.98 | 0.22 |
| No Dox | MCP2-Hs00180467_m1 | 1.00 | 0.83 | 1.21 | 35.76 | 16.02 | 0.28 |
| No Dox | MCP2L-Hs00389640_m1 | 1.00 | 0.86 | 1.17 | 25.54 | 7.11 | 0.22 |
| No Dox | NET1-Hs00610607_m1 | 1.00 | 0.66 | 1.17 | 32.94 | 4.16 | 0.22 |
| No Dox | NGEF-Hs00897849_m1 | 1.00 | 0.84 | 1.18 | 27.82 | 8.79 | 0.24 |
| No Dox | PLEKH2B-Hs00288156_m1 | 1.00 | 0.78 | 1.28 | 27.27 | 8.44 | 0.38 |
| No Dox | RHOA-Hs00357608_m1 | 1.00 | 0.66 | 1.17 | 24.83 | 3.09 | 0.22 |
| No Dox | TBP-Hs00999910_m1 | | | | 27.64 | | |
| No Dox | TGM1-Hs00180076_m1 | 1.00 | 0.70 | 1.43 | 36.36 | 17.51 | 0.51 |
| No Dox | TRIO-Hs00179278_m1 | 1.00 | 0.85 | 1.18 | 23.88 | 5.05 | 0.23 |
| No Dox | WAV1-Hs00232100_m1 | 1.00 | 0.61 | 1.20 | 30.67 | 11.77 | 0.31 |
| No Dox | WAV2-Hs00610104_m1 | 1.00 | 0.86 | 1.17 | 25.87 | 7.03 | 0.22 |
| No Dox | WAV3-Hs00196126_m1 | 1.00 | 0.85 | 1.18 | 26.66 | 10.02 | 0.26 |
| With Dox | 18S-Hs00999901_s1 | | | | 5.21 | | |
| With Dox | ABR-Hs00241607_m1 | 1.00 | 1.06 | 1.03 | 26.74 | 6.80 | 0.17 |
| With Dox | AKAP13-Hs00180747_m1 | 1.07 | 0.94 | 1.21 | 23.86 | 4.94 | 0.18 |
| With Dox | ARHGAP14-Hs00180327_m1 | 0.88 | 0.79 | 0.98 | 25.17 | 9.26 | 0.19 |
| With Dox | ARHGAP10L-Hs00737885_m1 | 1.20 | 0.91 | 1.77 | 30.38 | 16.48 | 0.45 |
| With Dox | ARHGAP11-Hs00267600_m1 | 1.08 | 0.73 | 1.53 | 24.27 | 5.94 | 0.63 |
| With Dox | ARHGAP12-Hs00229661_m1 | 1.03 | 0.93 | 1.13 | 23.66 | 5.04 | 0.16 |
| With Dox | ARHGAP15-Hs00299097_m1 | 1.14 | 0.96 | 1.37 | 30.85 | 17.96 | 0.20 |
| With Dox | ARHGAP17-Hs00237825_m1 | 0.80 | 0.63 | 1.12 | 26.76 | 7.63 | 0.40 |
| With Dox | ARHGAP18-Hs00248726_m1 | 1.00 | 0.85 | 1.25 | 25.04 | 6.80 | 0.20 |
| With Dox | ARHGAP24-Hs00190884_m1 | 0.93 | 0.81 | 1.08 | 27.08 | 8.17 | 0.19 |
| With Dox | ARHGAP24-Hs00218804_m1 | 1.34 | 1.16 | 1.53 | 27.48 | 9.57 | 0.20 |
| With Dox | ARHGAP4-Hs00209707_m1 | 1.04 | 0.91 | 1.18 | 30.82 | 11.98 | 0.18 |
| With Dox | ARHGAP5-Hs00180553_m1 | 1.14 | 1.02 | 1.28 | 26.78 | 7.66 | 0.15 |
| With Dox | BCR-Hs00244731_m1 | 1.00 | 0.88 | 1.32 | 26.24 | 7.33 | 0.31 |
| With Dox | EC22-Hs00216456_m1 | 1.07 | 0.91 | 1.12 | 23.97 | 5.00 | 0.15 |
| With Dox | FARP1-Hs00195010_m1 | 1.23 | 1.06 | 1.62 | 23.67 | 4.69 | 0.21 |
| With Dox | FLJ10357-Hs00218283_m1 | 1.17 | 0.98 | 1.42 | 26.78 | 6.68 | 0.29 |
| With Dox | GAPDH-Hs00999905_m1 | | | | 16.88 | | |
| With Dox | GEF1-Hs00611102_m1 | 0.48 | 0.44 | 0.54 | 31.82 | 15.01 | 0.16 |
| With Dox | MCP2-Hs00180467_m1 | 1.73 | 1.57 | 1.86 | 36.03 | 19.12 | 0.18 |
| With Dox | MCP2L-Hs00389640_m1 | 1.05 | 0.95 | 1.17 | 26.56 | 7.04 | 0.15 |
| With Dox | NET1-Hs00610607_m1 | 1.16 | 1.04 | 1.29 | 22.80 | 3.80 | 0.15 |
| With Dox | NGEF-Hs00897849_m1 | 0.88 | 0.76 | 1.07 | 27.88 | 9.97 | 0.19 |
| With Dox | PLEKH2B-Hs00288156_m1 | 0.78 | 0.62 | 0.98 | 27.76 | 9.78 | 0.33 |
| With Dox | RHOA-Hs00357608_m1 | 1.08 | 0.95 | 1.18 | 24.92 | 3.01 | 0.18 |
| With Dox | TBP-Hs00999910_m1 | | | | 27.64 | | |
| With Dox | TGM1-Hs00180076_m1 | 1.32 | 0.61 | 2.07 | 36.36 | 17.34 | 0.89 |
| With Dox | TRIO-Hs00179278_m1 | 1.13 | 1.01 | 1.28 | 23.78 | 4.67 | 0.18 |
| With Dox | WAV1-Hs00232100_m1 | 0.96 | 0.81 | 1.13 | 30.74 | 11.83 | 0.24 |
| With Dox | WAV2-Hs00610104_m1 | 1.00 | 0.90 | 1.10 | 25.95 | 7.04 | 0.15 |
| With Dox | WAV3-Hs00196126_m1 | 0.80 | 0.78 | 0.89 | 26.80 | 7.68 | 0.19 |

Supplementary Table 1:

HT29-Cdx2 cells were grown in absence or presence of Doxycycline for 7 days before RNA extraction. mRNA levels of 28 RhoA-GEFs, RhoA and 3 control genes (TBP, GAPDH, 18S) were analyzed by RT-qPCR using a custom 96-well plate containing specific TaqMan probes in triplicates. A representative experiment out of three is shown in which the RQs were derived from each Ct with the “NO Dox” condition being the calibrator.

Discussion

The homeotic transcription factor Cdx2 is required for intestinal homeostasis. In colon cancer, its expression is frequently down-regulated, especially in invasive cells (Subtil et al., 2007; Brabletz et al., 2004). This observation, together with experimental results that show that Cdx2 inhibits cell migration *in vitro* and metastasis formation in mice (Gross et al., 2008), strongly suggests that Cdx2 expression opposes metastasis formation in colon cancer. Here, we investigated potential mechanisms involved in the anti-metastatic activity of Cdx2 and found that Cdx2 impacts on the biomechanical properties of colon cancers cells, an activity that correlates with enhanced intercellular junctions and reorganization of the cytoskeleton through a Vav3-induced Rho signaling cascade.

1. Cdx2 promotes tissue rigidity through actin stress fibers and intercellular adhesion

During the metastatic process, it is likely that invasive cells have to acquire new biomechanical and biophysical features to detach from the primary tumor, invade and migrate through the surrounding tissue matrix. Accordingly, it has been observed that cells of various types of cancer are softer than normal cells (Li et al., 2008; Cross et al., 2007). In regard of the negative effect of Cdx2 on cell motility and metastasis formation, we examined for the first time the elasticity of a confluent monolayer of colon cancer cells using AFM and found that it was significantly decreased in the presence of Cdx2. This rigidity correlated with a clear remodeling of the Actin cytoskeleton into thick and parallel stress fibers. Different kinds of stress fibers have been described in motile and non-motile cells, showing that some stress fibers are more suited for motility and others for static contraction. In consequence, actin stress fibers can promote (Leve et al., 2011) or inhibit cancer cell migration (Vial et al., 2003). Here, it is likely that the observed stress fibers oppose cancer cell motility, as they appear in cells with increased rigidity and Cdx2-expressing cells proved to be less motile (Gross et al., 2008). Moreover, stress fibers are generally thicker in non-motile cells, which is conform to our observations in Cdx2-expressing cells (Pellegrin and Mellor, 2007). Another important observation was that E-cadherin is clearly reinforced at the cell membrane in Cdx2-expressing cells, also favoring increased cell adhesion. In line with this, it has recently been shown that Cdx2 increases the trafficking of E-cadherin to the cell membrane (Funakoshi et al., 2012). Given that both stress fibers formation and increased adhesion are associated with decreased motility and invasiveness of cancers cells (Vial et al., 2003; Pollock et al., 2005), it is likely that these processes are involved in the anti-metastatic effect of Cdx2.

2. Cdx2 activates RhoA, a GTPase regulating the cytoskeleton, and several Rho GEFs

To clarify how Cdx2 expression led to a reorganization of the Actin cytoskeleton, we focused on RhoA, since this small GTPase plays a key role in the formation of Actin stress fibers (Pellegrin and Mellor, 2007). Depending on the cell type, different effectors can mediate the activity of RhoA. Our data indicate that Cdx2 expression correlates with increased activity of Rho/MLC and Rho/Dia1 signaling cascades, respectively required for actomyosin contractibility and Actin nucleation (**Figure 8**). Interestingly, specific inhibition of RhoA did only reduce, not totally block the induction of stress fibers by Cdx2 (**Figure 3B**). Therefore, it is possible that also other, not yet identified, actors mediate stress fiber formation downstream of Cdx2. For example, a new Rho GTPase called Rif has been recently described to activate stress fiber formation via mDia and ROCK (Fan et al., 2010).

Similarly to other small GTPases, the activity of RhoA is stimulated by GEFs (guanine nucleotide exchange factors), which favor its GTP-bound active state by facilitating the exchange of GDP by GTP (Ridley et al., 2003). We found that mRNA levels of two Rho GEFs were indeed increased in cells expressing Cdx2, namely Vav3 and MCF2/Dbl. Further experiments with specific siRNA will be required to determine whether Vav3 and/or MCF2 actually mediate the effect of Cdx2 on stress fibers. Here, we focused on Vav3 regulation, as its expression was shown to be decreased in the intestines of Cdx2 Knockout mice (Gao and Kaestner, 2010). The shortest isoform of Vav3 showed the strongest stimulation by Cdx2 and was also found to be more expressed in the intestine or colon cancer cells lines (Zeng et al., 2000; Trenkle et al., 2000). It is likely that this short isoform is not generated by alternative splicing, but rather by the use of an alternative promoter (Trenkle et al., 2000). Accordingly, we were able to show that the 5' upstream regions of both isoforms were bound by Cdx2 in ChIP experiments and transcriptionally activated by Cdx2 in reporter assays (**Figure 7**). Yet, we failed to identify the Cdx2 binding sites, as mutation of all the potential sites we had found on the Vav3 promoters did not decrease transcriptional activation by Cdx2 (data not shown). One explanation is that Cdx2 does not directly bind to the Vav3 promoters, but associates with another transcription factor, a mechanism that has already been described for other Cdx2 target genes, such as Pept1 whose stimulation involves interaction with SP1 (Shimakura et al., 2006). However, the fact that neither the dominant negative isoform of Cdx2, nor the mutant Cdx2 proteins unable to bind DNA, could activate the Vav3 reporter, rather suggests a direct binding of Cdx2 to DNA, probably to less conserved binding sites.

3. Functional consequences of the Cdx2 / Vav3 connection in the intestine

The Vav family of proteins comprises in mammals three members of very peculiar Rho GEFs. Indeed, beside the typical PH (Pleckstrin-homology domain) and DH (DBL-homology domain) domains shared with other Rho GEFs, they harbor various others domains (namely a SH3-SH2-SH3 cassette), which allow them to act as adaptor molecules in several signaling pathways. Thus, Vav proteins can act independently of their function as Rho GEFs, to regulate various cell functions. Vav is essentially expressed in hematopoietic cell lineages and is critical for lymphocyte development (Fujikawa et al., 2003), whereas Vav2 and Vav3 show broader patterns of expression and may be involved in other biological functions. In particular, Vav3 is expressed in the intestine. Indeed, Munoz et al reported that Vav3 expression was enriched in Lgr5+ cells in the small intestine, suggesting that Vav3 may act in intestinal stem cells (Muñoz et al., 2012). In contrast, Liu et al only detected Vav proteins at the apex of differentiated cells, near adherens junctions, in the cecum and colon. Interestingly, mice invalidated for all Vav genes exhibited shorter differentiated cells in the cecum and colon, a defect that was linked to a reduced microtubular network, and mucosal ulcerations attributed to defective wound repair (Liu et al., 2009). Although Vav proteins were shown to induce drastic Actin reorganization in cell culture, the Vav triple Knockout mice did not seem to have altered Actin staining or distribution. This is contrast to Cdx2 conditional Knockout mice, which show absence of Actin bundling at the microvillus core of enterocytes (Gao and Kaestner, 2010). Thus, further investigation is clearly needed to determine the functional consequences of the Cdx2 / Vav3 connection in the intestine.

4. Role of the Vav3 isoforms

An additional level of complexity is brought by the fact that Vav3 encodes two very different proteins. Indeed, the large isoform, Vav3 v1, contains all the functional domains of Vav proteins, whereas the short isoform, Vav3 v2, consists only of the C-terminal SH3-SH2-SH3 domains (Figure 9), suggesting that the two proteins may exert different activities. This is strengthened by the fact that the corresponding transcripts exhibit distinct patterns of expression (Trenkle et al., 2000).

In particular, the short Vav3 isoform lacks the Rho binding domain (DBL-homology domain) and thus might not be able to act as a Rho GEF like the large isoform. On the contrary, it might even represent a dominant negative form of the full length Vav3, through competitive binding to activators or effectors. Alternatively, the short isoform might contribute to the activation of the large isoform, by acting via its SH2 and SH3 interaction domains as an adaptor or scaffold protein. Indeed, the simple up-regulation of Vav3 transcription is not sufficient to increase its activity, as it has to be

activated by tyrosine phosphorylation to relieve inhibition by the N-terminal region. Up to now, these possibilities have not really been explored experimentally. Similarly, because of the lack of specific antibodies against the short isoform, most of the analyses made on endogenous proteins did not discriminate both isoforms, which complicates their interpretation.

However, understanding the role played by the different Vav3 isoforms is important because Vav3 has been associated with cancer in various tissues. For instance, Vav3 is overexpressed in breast and prostate cancer compared to the benign tissue. In both cancer types, Vav3 stimulated cell growth and activated estrogen receptor (ER α) or androgen receptor (AR) via PI3-Akt signaling (Dong et al., 2006; Lee et al., 2008). Moreover, transgenic mice overexpressing Vav3 in the prostate developed chronic inflammation and tumors in this organ. Interestingly, the short Vav3 isoform had a dominant negative effect on the full length Vav3 isoform in prostate cancer cells and inhibited its induction of cell growth (Rao et al., 2012). In regard of these results, it would be important to compare the expression of the two Vav3 isoforms in the healthy intestine and in colon tumors, as well as their impact on colon cancer cells behavior, to understand if they mediate some of the anti-tumoral activities of Cdx2.

5. Conclusion

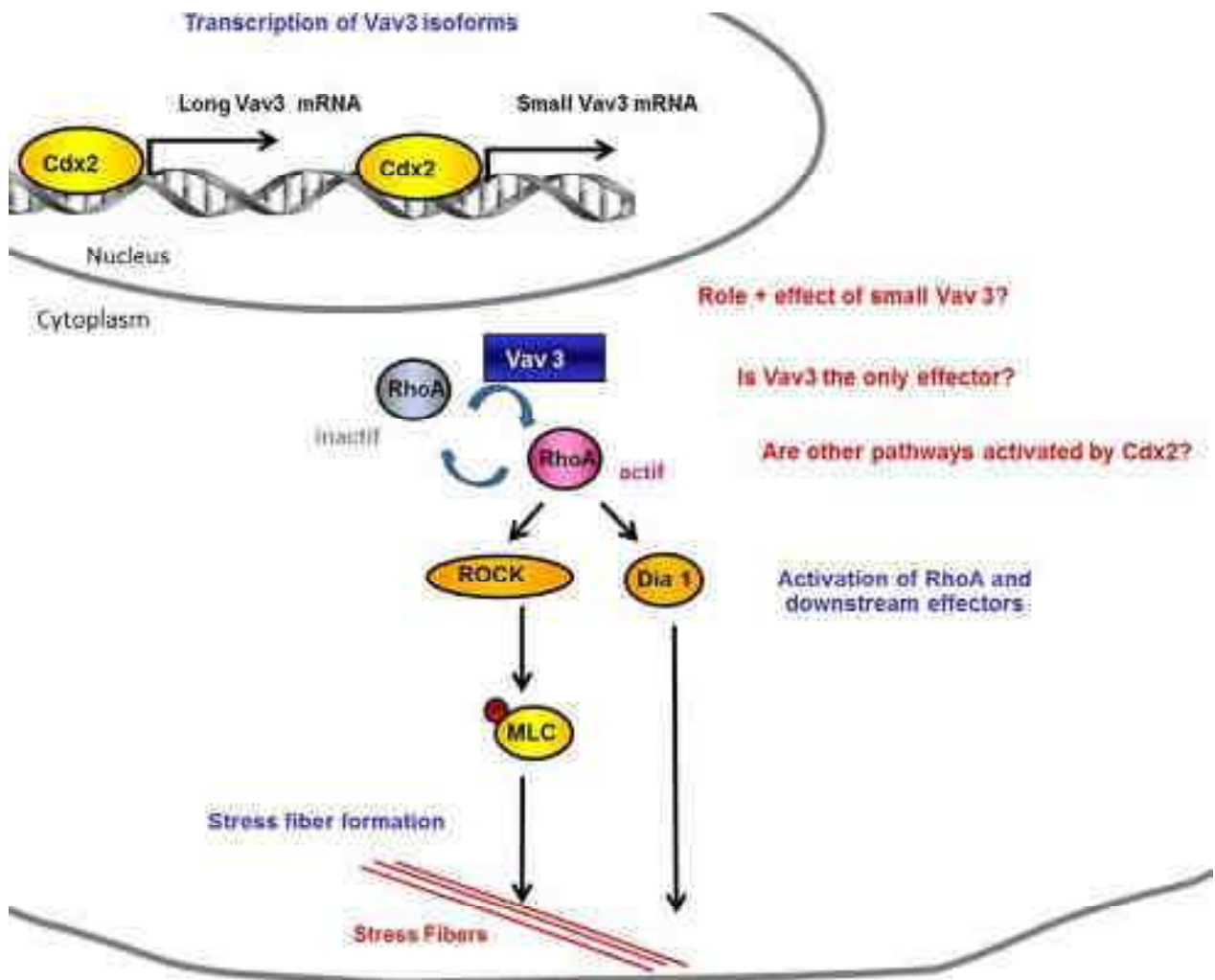


Figure 10: Principal results and remaining questions

In summary, we uncovered that Cdx2 favored tissue rigidity, an activity that might explain how Cdx2 opposes cell dissemination and metastasis. Moreover, we identified the Rho GEF Vav3 variants as new target genes of Cdx2 that may explain its activity on Rho signaling and Actin stress fibers formation. However, the role of the two Vav3 isoforms, as potential effectors of Cdx2, but also more generally in colon cancer cells remains to be elucidated.

PROJECT 3:

Determination of the molecular relays of Cdx2

Mucdhl, a new player in the intestinal homeostasis and colorectal cancer progression?

Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I. (2012). Cdx2 controls expression of the protocadherin Mucdhl, an inhibitor of growth and β -Catenin activity in colon cancer cells. *Gastroenterology*. Apr;142(4):875-885.e3. (Impact Factor =12) see Annex

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'"

Isaac Asimov
Science-fiction Writer and Biochemist. 1920-1992

Abstract

Background & Aims: The intestine-specific homeobox transcription factor Cdx2 is an important determinant of intestinal identity in the embryonic endoderm and regulates the balance between proliferation and differentiation in the adult intestinal epithelium. Human colon tumors often lose Cdx2 expression and heterozygous inactivation of Cdx2 in mice increases colon tumorigenesis. We sought to identify Cdx2 target genes, to determine how it contributes to intestinal homeostasis.

Methods: We used expression profiling analysis to identify genes that are regulated by Cdx2 in colon cancer cell lines. Regulation and function of a potential target gene were further investigated using various cell assays.

Results: In colon cancer cell lines, Cdx2 directly regulated the transcription of the gene that encodes the protocadherin Mucdhl. Mucdhl localized to the apex of differentiated cells in the intestinal epithelium and its expression was reduced in most human colon tumors. Overexpression of Mucdhl inhibited low-density growth of colon cancer cells and reduced tumor formation in nude mice. One isoform of Mucdhl interacted with β -catenin and inhibited its transcriptional activity.

Conclusions: The transcription factor Cdx2 activates expression of the protocadherin Mucdhl, which interacts with β -catenin and regulates activities of intestinal cells. Loss of Cdx2 expression in colon cancer cells might reduce expression of Mucdhl and thereby lead to tumor formation.

Introduction

Despite its complex architecture, the intestinal epithelium represents the most vigorously renewing adult tissue in mammals (Crosnier et al., 2006). Thus, dynamic temporal and spatial coordination of various cellular processes is not only required during fetal development of the gut, but also throughout adult life to maintain homeostasis.

Alterations of the signaling pathways that ensure controlled renewal of the gut are frequently found in gut pathologies, notably colorectal cancer (Markowitz et al., 2002) which corresponds to the second cause of cancer-related death in the Western world (Jemal et al.). This is well illustrated by the prominent loss of function of APC, which in particular regulates the protein levels of β -catenin: in addition to its adhesive function at the membrane, stabilized β -catenin relays in the nucleus the activity of the Wnt pathway which has a central role in the control of proliferation, cell stemness, differentiation and migration in the gut (Gross et al., 2008; Lai et al., 2009).

Another molecule required for the homeostasis of the gut and whose expression is frequently reduced in colon tumors (Subtil et al., 2007) is the homeodomain transcription factor Cdx2. Cdx2 specifies cell fate or axial position at different developmental stages (Beck et al., 1999) but a series of recent studies confirmed that Cdx2 is a major player in both the developing and the mature intestinal epithelium. Cdx2 expression in the embryo per se is detected around E8.5 and becomes progressively restricted to the intestinal epithelium at the adult stage (Guo et al., 2004). Endoderm-specific Cdx2 ablation from E9.5 onward results in colon agenesis, with the ectopic activation of a foregut/esophageal differentiation program reflecting an anterior homeotic transformation (Gao et al., 2009). Intestine-specific Cdx2 ablation around E14 is also lethal and leads to abnormal morphogenesis of the small intestine, with shortened villi and poorly differentiated, non-polarized, enterocytes (Gao and Kaestner, 2010). Strikingly, even intestine-specific loss of Cdx2 at the adult stage led to chronic diarrhea and death, with small intestine dilation and immature enterocytes, indicating that Cdx2 is required to maintain gut homeostasis throughout life (Verzi et al., 2011). Accordingly, reduction of Cdx2 expression promotes tumor progression in animal models of colon cancer (Aoki et al., 2003; Bonhomme et al., 2003), whereas forced expression of Cdx2 in colon cancer cells decreases metastasis in nude mice (Gross et al., 2008).

Altogether, these data correlate quite well with experiments performed *in vitro*. Indeed, forced expression of Cdx2 in intestinal cell lines enhances intestinal differentiation (Lorentz, 1997; Suh and Traber, 1996) and adhesion (Ezaki et al., 2007) while decreasing proliferation (Gross et al., 2008;

Mallo et al., 1998). On the opposite, silencing of Cdx2 disrupts apico-basal polarity (Gao and Kaestner, 2010) and enhances motility (Gross et al., 2008). On the molecular level, a certain number of genes regulated by Cdx2 have been identified, namely intestine-specific genes involved in the digestive function (Guo et al., 2004). As direct targets mediating the other functions of Cdx2 remain more elusive, we took advantage of microarray-based studies performed with intestinal cells showing modification of Cdx2 expression. One of the genes whose expression paralleled the one of Cdx2 was *Cdhr5*, also called *Mucdhl*. This gene encodes several splicing isoforms of a protocadherin mostly expressed in the kidney, liver and gut (Goldberg et al., 2000, 2002; Moulton et al., 2004). Protocadherins represent a group of less conserved members of the Cadherin superfamily which are membranous glycoproteins mediating Ca^{2+} -dependent cell-to-cell adhesion. All *Mucdhl* isoforms exhibit four cadherin extracellular motifs, but differ by the presence of several mucin-like tandem repeats located downstream of the cadherin motifs, a transmembrane domain and an intracellular domain containing many potential protein-binding motifs (Goldberg et al., 2000; Moulton et al., 2004). In this study, we investigated the regulation of *Mucdhl* expression in intestinal cells and initiated a comparative analysis of the function and mode of action of *Mucdhl* isoforms.

Materials and Methods

siRNA

Stealth/siRNA duplex oligoribonucleotides were from Invitrogen: CDX2HSS141544 (siRNA@Cdx2 544), CDX2HSS141546 (siRNA@Cdx2 546) and medium GC Duplex #2 as negative control.

Mucdhl-Expressing Cell Lines

The T-REx™ System (Invitrogen) was used to establish control and Mucdhl-inducible cell lines. First, HCT116TR cells, which constitutively express the tetracycline repressor, were established by transfection of HCT116 cells with pcDNA6/TR and blasticidin selection. Clone 7 of HCT116TR was transfected with pcDNA4/TO, pcDNA4/TO-DHLM/Flag or pcDNA4/TO-DHLL/HA and submitted to Zeocin selection. Several independent stable clones were obtained that expressed Flag-tagged Mucdhl M or HA-tagged Mucdhl L. These clones were maintained in DMEM supplemented with 10%FBS, Blastidicin (4µg/ml, Invitrogen), Zeocin (100µg/ml, Invitrogen) and antibiotics. Induction of expression was performed by adding doxycycline (1µg/ml, Sigma) to the medium.

Wound Healing and Grafting

Wound healing assay with Caco2/TC7 cells and grafting of HT29 cells were described (Gross et al., 2008; Benahmed et al., 2007). Sub-cutaneous injections of HCT116 Mucdhl stable cells (1.10^6 cells/each side) in female nu/nu mice (Janvier) were performed similarly, with half of the mice receiving doxycycline (400 µg/ml) in their drinking water described (Gross et al., 2008). Mice were killed (clone M: 8 weeks; clone L: 4 weeks) and tumors measured.

Transfections

Plasmid transfections were performed with Lipofectamine 2000 (Invitrogen) or Jetprime (Polyplus Transfection), except for Luciferase assays in which JetPEI (Polyplus Transfection) was used. siRNA duplexes were transfected with Lipofectamine RNAiMAX (Invitrogen).

RNA Extraction and RT-PCR

Total RNA was isolated using TRI Reagent (MRC).

Semi-quantitative RT-PCR were performed as described (Gross et al., 2008) with the following primer pairs: hMucdhl L (F: CTC CCA CCA ACC AAC CAC; R: CAT ATC CAC CAC CGA GAA GC), hMucdhl M (F: TGG AGG GAG AGG TTG TGC T; R: GGC CGC CAC CTG TGG AGG), hCdx2 (F: AAG CCG AGC TAG CCG CCA CGC TGG; R: TGA GAG CCA GGT CTG TAG GTC TAT) and as internal control hTBP (F: CGT GTG AAG ATA ACC CAA G; R: ATT GGA CTA AAG ATA GGG A).

Luciferase Assays

Luciferase assays were performed with the Dual-Luciferase Reporter Assay (Promega) as described (Gross et al., 2008). Data presented correspond to a representative experiment performed in triplicate.

In Situ Proximal Ligation Assay (PLA)

Cells seeded in 8-well CultureSlides (BD Falcon) were transfected and treated overnight with MG132 (10 μ M, Sigma) before fixation (PFA 4%, 15 min) and permeabilization (Triton X-100 0.5% in PBS, 30 min). Slides were processed for in situ PLA with the Duolink[®] II Detection Reagents Green, Duolink[®] II PLA probe anti-Mouse PLUS and Duolink[®] II PLA probe anti-Rabbit Minus (Olink Bioscience) as recommended. Primary antibodies were: rabbit anti- β -catenin (clone 6B3, CST; 1/150) and mouse anti-Flag (clone M2, Sigma; 1/500). To check the specificity, control experiments omitting one of the primary antibodies were performed. Imaging was based on optical sectioning using the ApoTome system.

Colony Formation and Soft Agar Assays

For colony formation assays, 250 cells in 1ml of medium were added to 1ml of medium without or with doxycycline (2 μ g/ml) per well of a 6-well plate (3 replicates) and allowed to grow for 14 days before fixation (70% Ethanol), staining with Crystal violet (0.05% in PBS) and counting of the colonies. For soft agar assays, cells were mixed with DMEM containing 10% FBS and 0.4% low melting point agarose (Promega). 1.5ml of this solution (5000 cells) were added to each well of a 6-well plate previously coated with 1.5ml of 0.6% low melting agarose (3 replicates). After 30 min, 1ml of growth medium without or with doxycycline (1 μ g/ml) was added per well. Medium was changed every 2-3 days. After 3 weeks, colonies were fixed, stained and counted.

Statistics

* $p < 0.01$ and ** < 0.001 given by a one-way ANOVA test followed by a Newman-Keuls test.

Cell Lines

Caco-2/TC7 (Chantret et al., 1994) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 20% heat-inactivated Fetal Bovine Serum (FBS, Invitrogen), 1% essential amino-acids and antibiotics. Human colon adenocarcinoma HCT116, HT29, T84 and SW480 cells (ATCC) and embryonic kidney HEK293 (ATCC) were grown in DMEM supplemented with 10% FBS and antibiotics.

Plasmids

pRL-null was from Promega and pEGFP-C1 from Clontech. pcDNA6/TR and pcDNA4/TO were from Invitrogen. pTOPFLASH and pFOPFLASH (Korinek et al., 1997), pcDNA1 expressing Myc-tagged TCF4 and pCI-Neo expressing human β -catenin (WT or S33Y) were from H. Clevers (Utrecht University Medical Center, The Netherlands). pcDNA3 plasmids expressing Flag-tagged human β -catenin (WT or S33Y) were from Addgene (#16828 and #19286, (Kolligs et al., 1999)).

pcDNA4/TO-DHLM/Flag and pcDNA4/TO-DHLL/HA allow inducible expression of C-terminally Flag- or HA-tagged human Mucdhl M (NP_112554.2) and Mucdhl L (NP_068743.2) respectively. To generate pcDNA4/TO-DHLM/Flag, a Sall-BglII-digested PCR fragment of 2026 bp was first obtained using the PCRx Enhancer System (Invitrogen), a Mucdhl M-containing plasmid (clone IRATp970E11112D, Imagen) as template and Mucdhl M specific primers including the sequence for the Flag tag (TTG CTG AGA TCT CTG CCA GGT GAC AGC CGC CAA GATG and GCT AGC GCG TCG ACT TAC TTG TCG TCT TTG TAG TCG ATG TAG GAG TCA TCA CCA CCG G) and then sub-cloned into BamHI / XhoI of pcDNA4/TO. To generate pcDNA4/TO-DHLL/HA, a fragment containing part of human Mucdhl L coding sequence (post M/L splicing site) obtained by RT-PCR using Caco2/TC7 RNA and Mucdhl L specific primers including the sequence for the HA tag (CTG ACC ACC ACC ACA CTG GCA CAG GCG and TTA TCT AGA TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA GAT GTA GGA GTC ATC ACC ACC GGG CGC) was first sub-cloned into pCRII/TOPO (Invitrogen): a 1310 bp BstXI-XbaI fragment from the resulting construct was switched with the 731 bp BstXI-XbaI fragment of pcDNA4/TO-DHLM/Flag to generate pcDNA4/TO-DHLL/HA. To obtain pcDNA4/TO-DHLM/HA, the 587 bp XbaI-AfeI fragment of pcDNA4/TO-DHLL/HA containing the HA tag sequence was switched with the corresponding fragment in pcDNA4/TO-DHLM/Flag.

pFLAG-hCdx2 was obtained by first sub-cloning a RT-PCR fragment containing Cdx2 into pCRII/TOPO (Invitrogen) and next inserting a HindIII-XbaI fragment from the resulting plasmid into pFLAG-CMV2 (Sigma).

To obtain wild-type pMucdhl-Luc constructs, a 2.8 and a 1.4 kb DNA fragments generated by PCR using T84 cells genomic DNA, the PCRx Enhancer System (Invitrogen) and Mucdhl specific primers including the sequence for NheI and KpnI restriction sites (2.8 kb F: TAT CGG TAC CCC TGA CCT TCC CGG GAT CGC T; 1.4 kb F: TAC AAG GTA CCA AGG AGG AAT CAG ACC CAG ACG; R both: TAC TAG CTA GCT TGG CGG CTG TCA CCT GGC AG) were first sub-cloned into pCRII/TOPO (Invitrogen) and next transferred into pGL3-basic (Promega) using KpnI and NheI. Mutated pMucdhl-Luc plasmids were generated with the Quikchange II XL Site-Directed Mutagenesis Kit (Stratagene) using first pMucdhl(1.4 kb)-Luc as

template: m1-pMucdhl(1.4 kb)-Luc exhibited a mutation of the most proximal Cdx2 putative binding site (- 105: ATAAA switched to GTGCA using GAA GCC GGT CGT AAA GGA TCG TGC ACT GGG CGG CGT CTG and CAG ACG CCG CCC AGT GCA CGA TCC TTT ACG ACC GGC TTC primers) and m2-pMucdhl(1.4 kb)-Luc exhibited a mutation of the less proximal Cdx2 putative binding site (- 114, TAAA switched to AGTA using CTT GGC AGA AGC CGG TCG AGT AGG ATC ATA AAC TGG GCG and GAA CCG TCT TCG GCC AAC TCG TCC TAG TAT TTG ACC CGC primers). m1m2-pMucdhl(1.4 kb)-Luc which is mutated on both Cdx2 consensus binding sites was generated by using m1-pMucdhl(1.4 kb)-Luc as template and the m1m2 mutagenesis primer pair (TGG CAG AAG CCG GTC GAG TAG GAT CGT GCA CTG GG and CCC AGT GCA CGA TCC TAC TCG ACC GGC TTC TGC CA). Each new construct was checked by sequencing (GATC Biotech).

RT-qPCR And Microarray Analysis

For RT-qPCR, reverse transcription (RT) was performed in 20 µl using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and 2 µg of RNA. Diluted RT products (5-10x) were mixed with TaqMan Master Mix (Applied Biosystems) and gene-specific TaqMan probe and primers sets (TaqMan Gene Expression assays, Applied Biosystems) to perform quantitative PCR (qPCR) using the 7500 real-time PCR System (Applied Biosystems). The following TaqMan Gene Expression assays were used: Mucdhl, Hs01059246_m1 (detecting both isoforms); Cdx2, Hs00230919_m1; TBP, Hs99999910_m1; Sl, Hs00356112_m1). Analysis of the results (duplicates) was performed with the 7500 software v2.0.1 (Applied Biosystems) using the relative $\Delta\Delta C_t$ quantification method.

Exploratory microarray-based experiments were performed in triplicates at the Microarray and Sequencing Platform of the IGBMC (Strasbourg, France) using spotted high-density human 25K oligonucleotide arrays (Le Brigand et al., 2006).

Chromatin Immunoprecipitation (ChIP)

Fixation of HCT116 cells was performed with 1% (v/v) formaldehyde for 10 min at room temperature and quenched with 0.125 M Glycine for 5 min. ChIP experiments were carried out using the EZ-Magna ChIP™ G Chromatin Immunoprecipitation kit as recommended by the supplier (Upstate Cell Signaling). Sheared crosslinked chromatin from $\sim 10^6$ cells was incubated overnight at 4°C with 1 µg of mouse IgG (Upstate Cell Signaling) or with mouse anti-Cdx2 antibody (Biogenex). Input corresponds to non-immunoprecipitated sheared crosslinked chromatin from $\sim 10^5$ cells (1%). PCR analysis was performed with Taq PCRx DNA Polymerase (Invitrogen), 1/25th of immunoprecipitated DNA as template and primers located in the human Mucdhl promoter (F: ACC CAT GAG CCG CTC TCC AGT C; R: TCT GGC GTC TAG GAC TGG CGC AGT; annealing: 55°C; product: 248 bp) or the Sl promoter (F: GGC TGG TAA GGG TGC AAT AA; R: GCC TGT TCT CTT TGC TAT GTT G; annealing: 55°C; product: 119 bp).

Immunoprecipitation and Western blot

Cells were treated or not with MG132 before lysis in 2% NP40-150mM NaCl-50mM Tris pH7.5 lysis buffer containing protease inhibitors (Complete, Roche). For exogenous immunoprecipitation, 1 mg of cellular extract was incubated 2h at 4°C with 30µl of anti-HA affinity matrix (Roche). For endogenous immunoprecipitation, 1mg of cellular extract was incubated O/N at 4°C with 1µg of antibody β -catenin (clone 14, BD Biosciences) or mouse Ig (Chemicon) and 30µl of protein G-Agarose beads (Roche) were added the last 45 min. In both cases, beads were next washed 4 times for 10 min with lysis buffer adjusted to 500mM NaCl, resuspended in 20µl of Laemmli loading buffer (Euromedex) and boiled for 5 min before SDS-PAGE.

Western blot analysis was performed as described (Gross et al., 2005) with one of the following primary antibodies: Cdx2 (Biogenex; 1/5000), β -catenin (Clone 14, BD Biosciences; 1/5000), Flag (clone M2, Sigma; 1/2000), Gapdh ((Launay et al., 1989); 1/3000), Mucdhl (H-225, Santa Cruz; 1/3000), Mucdhl (C-20, Santa Cruz; 1/500) and Actin (Chemicon International; 1/15000).

Immunohistochemistry and immunofluorescence

H&E staining and immunohistochemistry were performed as described (Gross et al., 2008) using anti-Cdx2 (Biogenex; 1/500), anti-Mucdhl (H-225, Santa Cruz; 1/500) or anti- β -catenin (Clone 14, BD Biosciences; 1/200) antibody. Murine tissues were from WT, APC ^{Δ 14/+} (Colnot et al., 2004) or Cdx2^{+/-} (Beck et al., 1999) mice, treated or not with AOM as described (Bonhomme et al., 2003). Human samples were from the collection of the University Hospital of Strasbourg (France).

Immunofluorescence was performed on fixed (4% PFA, 15 min), paraffin-embedded sections of soft agar-grown colonies. After antigen-retrieval (10 min in 10mM Sodium citrate pH6), slides were blocked (5% NGS-0.1% Triton-PBS, 1h at RT°), incubated O/N at 4°C with diluted primary antibodies (1/250e Mucdhl H-225 from Santa Cruz and 1/125e β -catenin Clone 14, from BD Biosciences), washed 3 times for 5 min with 0.1% Triton-PBS, incubated 1h at RT with diluted secondary antibodies (1/1000e Alexa Fluor 488 goat anti-mouse IgG and 1/1000e Alexa Fluor 568 donkey anti-rabbit IgG), washed again, stained for DAPI (Sigma) and mounted with FluorSave Reagent (Millipore). Imaging was based on optical sectioning using the ApoTome system (Zeiss) and the ImageJ software.

Deglycosylation

Cells were lysed in 2% NP40-150mM NaCl-50mM Tris pH7.5 lysis buffer containing protease inhibitors (Complete, Roche). Deglycosylations were performed at 37°C on 10µg of protein extract. To remove N-glycosylation, extracts were incubated 2h with PNGase F (native, NEB) as recommended. To remove both N and O-glycosylation, extracts were in addition treated overnight with Neuraminidase and Endo-a-N-Acetylgalactosaminidase (both NEB) as recommended.

Proliferation in Standard Conditions

Growth of cell population in standard conditions (-/+ doxycycline) was evaluated by performing MTS assays (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) at different time points after seeding 10000 cells/well in 96-well plates on day 1 (6 replicates).

Results

1. Expression of Mucdhl depends on the presence of Cdx2 in intestinal cells

We previously reported similar changes in mRNA expression and/or protein distribution during *in vitro* wound healing and tumor dissemination in colon cancer for several molecules. In particular, β -catenin accumulated in the nucleus of migrating cells at the wound edge or at the invasive front of tumors, while Cdx2 expression was down-regulated in both situations (Gross et al., 2008). To obtain a global view of the transcriptional events occurring, we performed exploratory gene-profiling analysis on Caco-2/TC7 cells located either at the wound edge or in the confluent monolayer. One of the genes whose expression was predicted to be down-regulated in migrating cells was Mucdhl. Strikingly, the expression of Mucdhl was also modified in another microarray analysis in which transcriptomes from HT29 colon cancer cells grown *in vitro* or grafted in nude mice (an experimental set-up that revealed the influence of the micro-environment on Cdx2 expression) were compared (Benahmed et al., 2007). As in both cases Mucdhl and Cdx2 expressions were similarly modified, we hypothesized that Mucdhl might be a transcriptional target of Cdx2. First, we verified the data of the microarrays by RT-PCR for the two major isoforms of Mucdhl detected in intestinal cells, Mucdhl M and Mucdhl L (**Figure 1A-C**). Second, we observed that, similarly to Cdx2, expression of Mucdhl rises during spontaneous differentiation of Caco-2/TC7 intestinal cells grown at confluence (**Figure 1D** and **Supplementary Figure 1**).

Next, we performed direct gain and loss of function experiments to confirm that Mucdhl expression depends on Cdx2. When Cdx2 was overexpressed in colon (HCT116, SW480) or renal (HEK293) cell lines, Mucdhl expression determined by quantitative RT-PCR (RT-qPCR) was increased (**Figure 1E**). RT-PCR using isoform-specific primers confirmed that the augmentation affected both isoforms (not shown). When Cdx2 expression was silenced with two different siRNA in SW480 cells, Mucdhl expression was concomitantly decreased (**Figure 1F**). Thus, Cdx2 not only enhances, but is also required for Mucdhl expression in intestinal cells.

2. Mucdhl is a transcriptional target of Cdx2

Examination of the 3 kb DNA region upstream of the coding sequence of Mucdhl revealed the presence of potential Cdx2-binding sites. To determine if Cdx2 is able to bind to the Mucdhl promoter, we performed chromatin immunoprecipitation (ChIP) experiments in HCT116 cells, which express low levels of endogenous Cdx2 and Mucdhl: upon overexpression of Cdx2, a DNA fragment corresponding to the Mucdhl promoter was amplified by PCR in Cdx2 immunoprecipitates

(**Figure 2A**). Similarly, ChIP performed with differentiated Caco-2/TC7 extracts revealed the binding of endogenous Cdx2 to the Mucdhl promoter (**Figure 2B**).

To check if Cdx2 regulates the transcription of Mucdhl, we generated two luciferase reporter plasmids, which differ by the size of the Mucdhl promoter region and thus the number of potential Cdx2-binding sites (**Figure 2C**). When transfected into HCT116 cells, the luciferase activity of these pMucdhl-Luc reporters was stimulated by Cdx2 (**Figure 2D**). Both reporters exhibited similar activation, suggesting that the required elements were contained within the 1.4 kb region upstream of the Mucdhl translation site. To determine if any of the two Cdx2 binding sites present in this region was involved, we generated by site-directed mutagenesis additional pMucdhl-Luc reporters, harboring only one of the Cdx2-binding sites or none (**Figure 2C**): activation of the mutated pMucdhl-Luc reporters by Cdx2 was slightly reduced by the loss of the m2 site, severely decreased by the m1 site and abolished by the loss of the two sites (**Figure 2E**). Thus, both sites seem to be involved, with the m1 playing a prominent role, consistent with the fact that its sequence is a better match of the consensus Cdx2-binding site. Similar results were obtained in SW480 cells (not shown). Altogether, this set of data indicates that Cdx2 directly regulates the transcription of Mucdhl by binding to its two proximal Cdx2 consensus sites.

3. Mucdhl expression in the intestinal epithelium is reduced upon loss of Cdx2 and tumorigenesis

In murine embryos, Mucdhl was detected by immunohistochemistry in the gut around E16.5, concomitant to intestinal cytodifferentiation (**Figure 3A**). In the mouse and human adult small intestine, Mucdhl was predominantly observed in the differentiated cells of the villi, in particular at the brush border (**Figure 3B** and **Supplementary Figure 2**). Expression was mainly detected at the plasma membrane, especially at the apex of the cells, and weakly in the cytoplasm. In the colon, Mucdhl exhibited an increasing gradient towards the surface of the epithelium, indicating that Mucdhl was more expressed in differentiated cells. In addition, consistent with the role of Cdx2 in Mucdhl expression, Mucdhl is lost in foregut-type heterotopia appearing in the peri-caecal lesions of Cdx2^{+/-} mice (**Figure 3C**) and totally lacking Cdx2 expression (Beck et al., 1999).

Finally, we examined Mucdhl expression in colon tumors. Mice treated with the AOM carcinogen or tumor-prone (APC^{Δ14/+ 24}) exhibited colon tumors with reduced levels of Cdx2 and Mucdhl (**Figure 4A**). In human colon tumors, Mucdhl expression was also frequently lower than in the normal epithelium, as shown by immunohistochemical staining and RT-qPCR (**Figure 4B, C**).

4. Mucdhl can reduce colony and tumor formation of colon cancer cells

To investigate the function of Mucdhl, we established stable cell lines expressing Mucdhl upon doxycycline in HCT116 cells, which normally produce only trace amounts of endogenous Mucdhl (**Figure 5A**). For both isoforms, and as previously reported (Goldberg et al., 2000), multiple bands were detected by Western blot, which reflects post-translational modifications, mainly glycosylation (**Supplementary Figure 3**).

Growth of control and Mucdhl-expressing clones cultivated in standard conditions was equivalent (**Supplementary Figure 4**). In contrast, Mucdhl M clearly reduced the number of colonies in a colony formation assay (**Figure 5B**). The same effect was observed with Mucdhl L, although to a lower extent and with more variability between the clones, but was not seen in control clones. In addition, the few colonies that formed in the presence of Mucdhl appeared smaller and disorganized (**Figure 5B**, inset). Colony formation was also examined in anchorage-independent conditions by plating the cells in soft agar or matrigel (not shown). Again, the number of colonies was reduced by Mucdhl, especially by Mucdhl M, which almost totally abolished colony formation (**Figure 5C**). Even when cells were allowed to form colonies in soft agar for seven days before addition of doxycycline, expression of Mucdhl still led to fewer, smaller and more disorganized colonies (**Figure 5D**).

To determine the impact of Mucdhl on tumor formation *in vivo*, control and Mucdhl-expressing cells were injected under the skin of nude mice. Expression of either Mucdhl isoform led to smaller sub-cutaneous tumors (**Figure 6**), indicating that both Mucdhl M and L can oppose tumor formation *in vivo*.

5. Mucdhl M can interact with β -Catenin and reduce its transcriptional activity

As several members of the cadherin superfamily interact with β -catenin, we checked if this was the case for Mucdhl. Co-immunoprecipitation experiments were performed with extracts from HEK293 cells transfected with various combinations of expression vectors for β -catenin and the Mucdhl isoforms. As shown on **Figure 7A**, β -catenin (wild-type or S33Y stabilized mutant (Kikuchi, 2000)) co-immunoprecipitated with Mucdhl M. In contrast, β -catenin was barely detectable in immunoprecipitates of Mucdhl L. Similar results were obtained in HCT116 and SW480 cells (not shown). We also performed co-immunoprecipitation experiments with Caco-2/TC7 cell extracts to detect endogenous interaction: indeed, in β -catenin immunoprecipitates from differentiated Caco-2/TC7, the Mucdhl antibody detected a band of 130 kDa corresponding to the size of one of the Mucdhl M bands (**Figure 7B**). The presence of β -catenin and Mucdhl M in the same protein complex

was further confirmed using in situ PLA (Proximal Ligation assay (Söderberg et al., 2008)), a technique that detects protein interactions within intact cells by immunofluorescence: as shown on **Figure 7C**, co-expression of Mucdhl M and β -catenin in HEK293 cells generated a specific PLA signal that was slightly enhanced at the membrane (same results with HCT116, SW480 cells: not shown).

These results prompted us to examine if Mucdhl M expression had any impact on β -catenin sub-cellular localization, which is critical for its activity. In cells expressing transiently Mucdhl M, we failed to detect a consistent change in the localization of β -catenin by immunostaining or sub-cellular fractionation (**Supplementary Figure 5**). However, in HCT116 stable cells expressing Mucdhl M and grown in soft agar, the membrane localization of β -catenin appeared more pronounced (**Figure 7D**).

Finally, we examined if this physical interaction interfered on the transcriptional activity of β -catenin. We performed luciferase assays, using the pTOPFLASH reporter that reflects nuclear activity of β -catenin (Korinek et al., 1997) In SW480 cells, which exhibit high constitutive activity of β -catenin due to the loss of APC function, expression of Mucdhl M, but not Mucdhl L, reduced the activity of pTOPFLASH and had no effect on the control reporter pFOPFLASH (**Figure 7E** and **Supplementary Figure 6**). In H293 cells, activity of pTOPFLASH induced by TCF4 and a stabilized β -catenin was clearly diminished by Mucdhl M, and weakly by Mucdhl L (**Figure 7F**). Altogether, these data show that the interaction of Mucdhl M with β -catenin results in decreased β -catenin transcriptional activity.

Figures

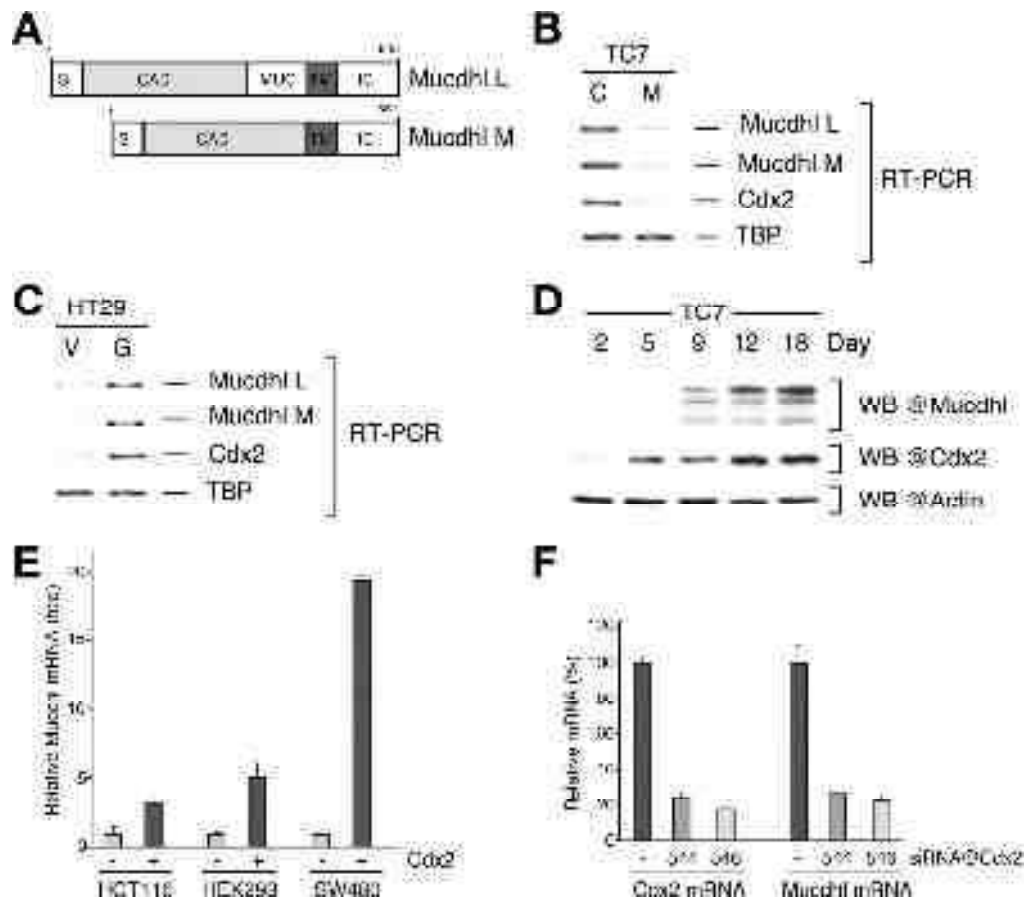


Figure 1: Mucdhl expression depends on Cdx2

(A) Schematic representation of human Mucdhl L and M (S: signal peptide; CAD: cadherin motifs; MUC: mucin-like tandem repeats; TM: transmembrane domain; IC: intracellular domain). **(B)** A monolayer of Caco-2/TC7 cells was wounded and 48h later cells located at the wound edge (M) or in the confluent monolayer (C) were separately collected for RNA extraction and RT-PCR with TBP as internal control. **(C)** HT29 were cultivated *in vitro* (V) or sub-cutaneously grafted (G) before RNA extraction and RT-PCR. **(D)** Caco-2/TC7 cells were grown for the indicated time before Western blot analysis. The multiple bands detected by the anti-Mucdhl antibody reflect as reported the presence of several isoforms and glycosylation (Goldberg et al., 2000, 2002; Moulton et al., 2004). **(E)** Cdx2 was overexpressed (+) by transfection before RNA extraction and RT-qPCR. Results are presented as fold of induction for each cell line, with the level of Mucdhl expression (-/+ SD, normalized with TBP expression) in cells without Cdx2 overexpression set at 1. **(F)** SW480 cells were transfected with control (-) or Cdx2-specific siRNAs before RNA extraction and RT-qPCR. Results are presented as % of expression with the level of Cdx2 or Mucdhl expression (-/+ SD, normalized with TBP) in the control sample set at 100%.

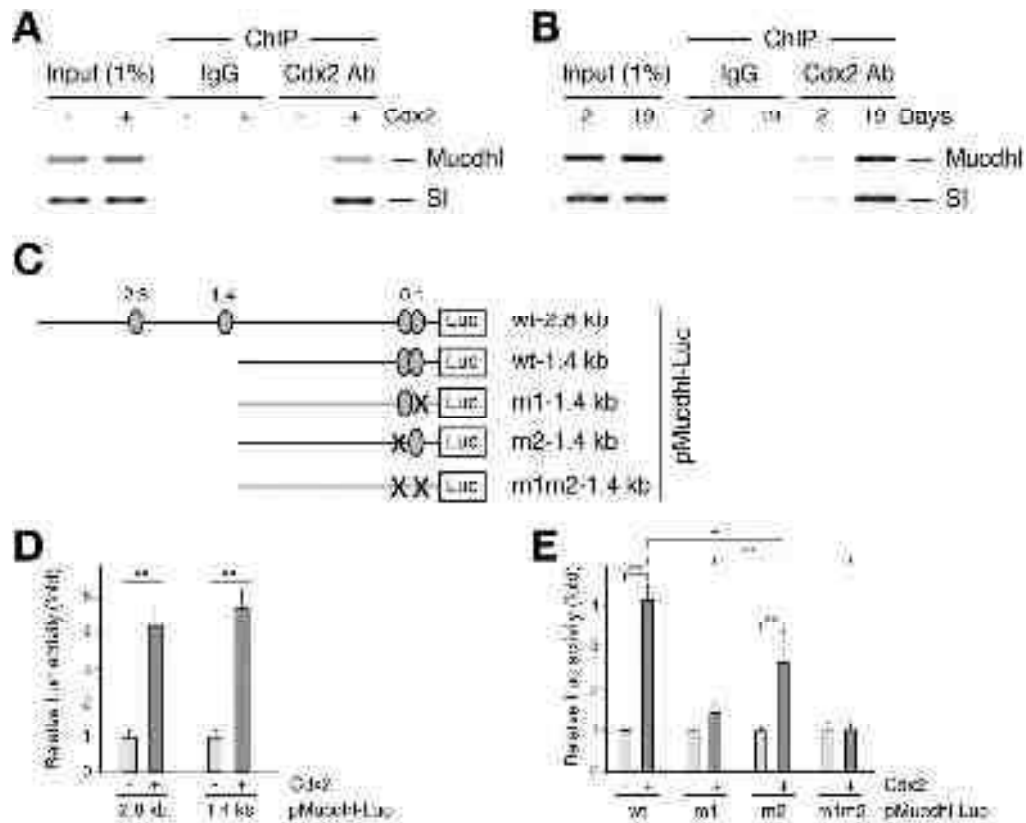


Figure 2: Cdx2 regulates the transcription of Mucdhl

(A) Chromatin of HCT116 cells expressing Cdx2 (+) or not (-) was subjected to immunoprecipitation (ChIP) with an anti-Cdx2 antibody or control IgG before PCR detection of a Mucdhl or a Sucrase-Isomaltase (SI, positive control) promoter fragment. PCR was also performed with 1% of the bulk DNA (input) to ensure equivalence of the samples. **(B)** As in (A) with Caco2/TC7 grown for 2 or 19 days. **(C)** Schematic representation of the different Mucdhl reporter plasmids tested with the positions of the potential Cdx2-binding sites (intact: O; mutated: X). **(D)** HCT116 were transfected with a Cdx2-expressing plasmid (+) or the corresponding empty plasmid (-), the indicated pMucdhl-Luc reporter plasmid, as well as a promoter-less renilla reporter plasmid (internal control), before lysis and luminescence measurement. Relative promoter activity (-/+ SD, normalized with renilla values) of each of the pMucdhl-Luc reporter in the absence of Cdx2 was set at 1. **(E)** As in (D), using the indicated reporter plasmids.

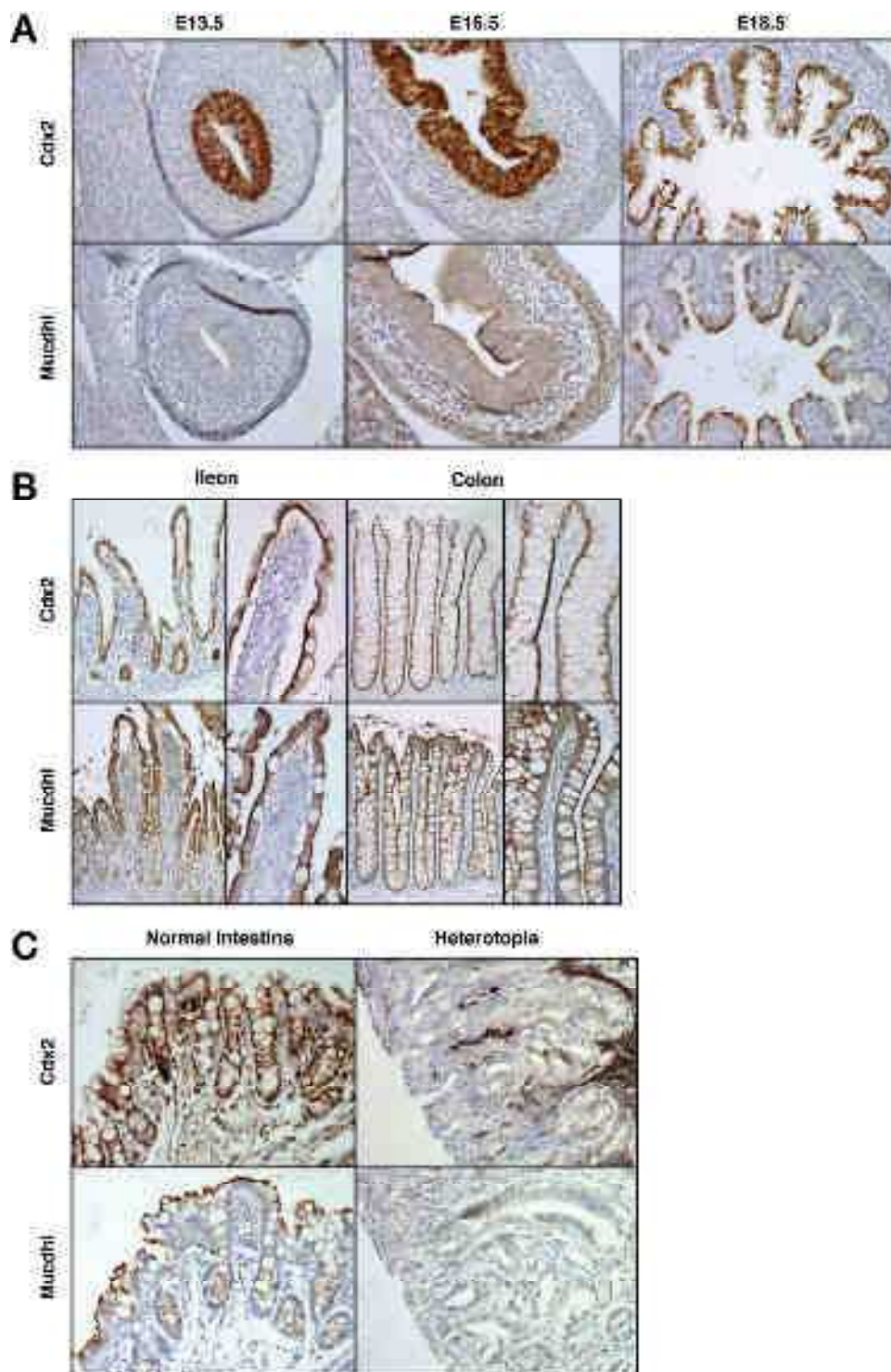


Figure 3: Expression of Mucdhl in wild-type and Cdx2^{+/-} intestines

Cdx2 and Mucdhl antibodies were used on paraffin-embedded sections. **(A)** Staged mouse embryos. **(B)** Adult human intestines. **(C)** Matching areas of normal intestinal epithelium and foregut-type heterotopia of a Cdx2^{+/-} mouse. Original magnification 200X except in (B) with 100X and 200X.

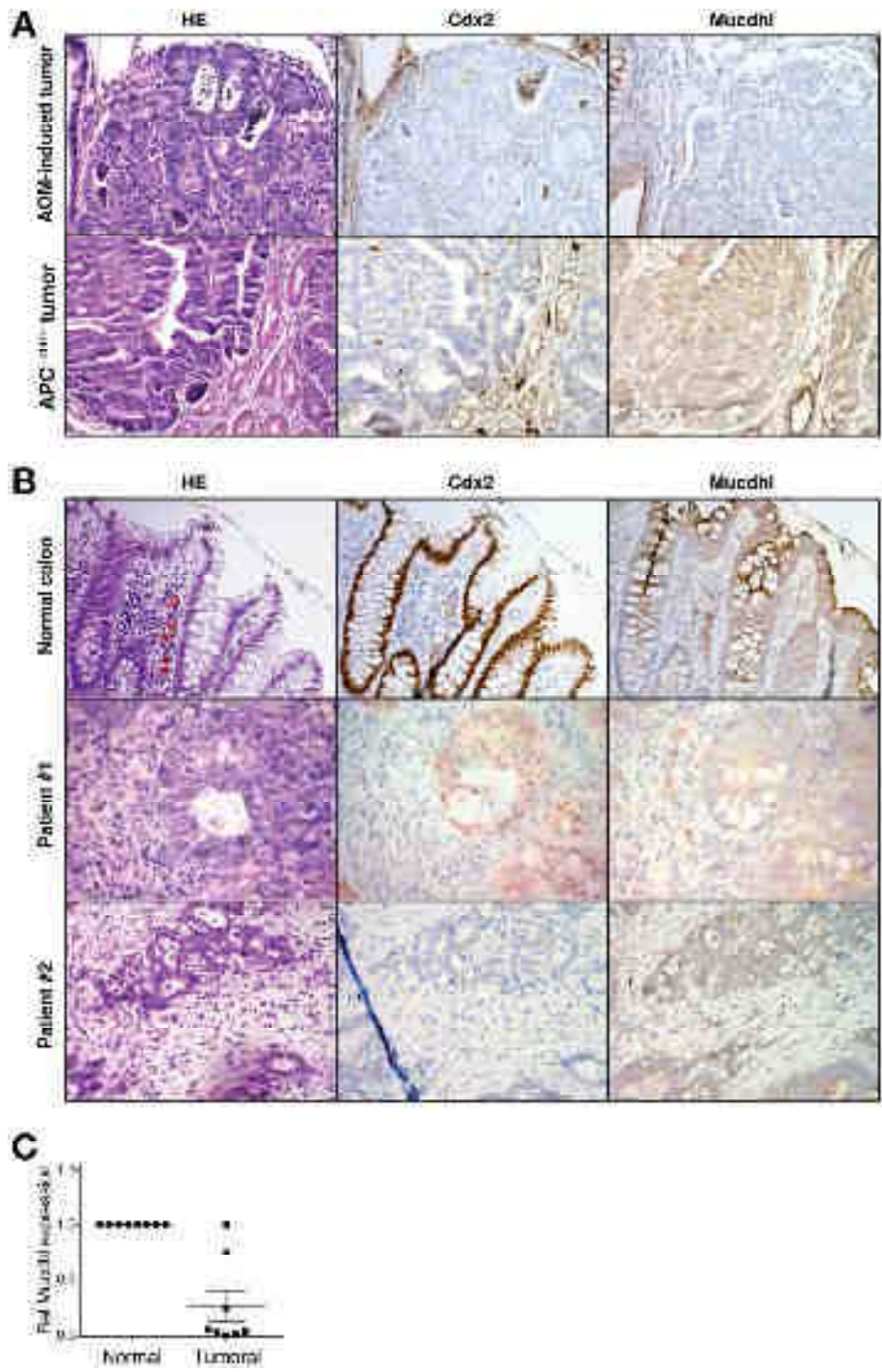


Figure 4: Mucdhl expression is reduced in colon tumors

(A) H&E staining and immunohistochemistry (IHC) were performed on tumor tissue from the distal colon of AOM-treated mice or APC^{Δ14/+} mice. (B) H&E and IHC were performed on normal colon and colon tumors from two patients (original magnification 200X). (C) RT-qPCR were performed on RNA from matched normal (dots) or tumor (squares) colon tissues. Relative Mucdhl expression (-/+ SEM, normalized with TBP) for each normal colon was set at 1.

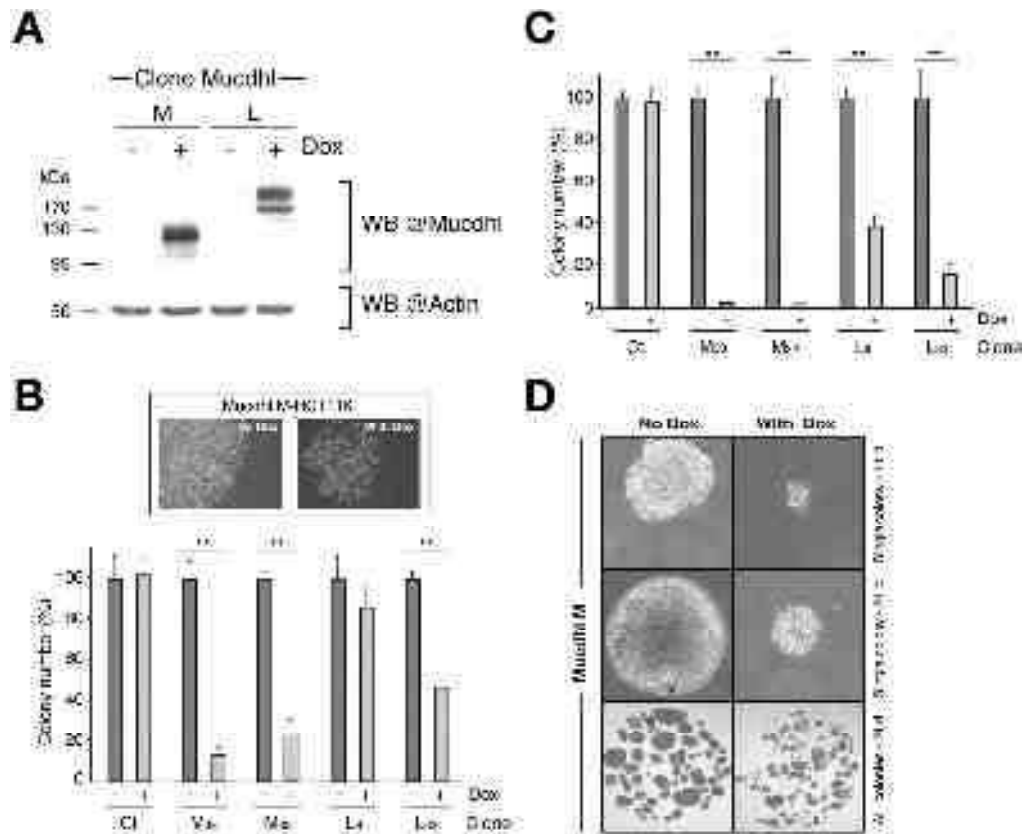


Figure 5: Mucdhl can reduce colony formation of colon cancer cells

(A) Western blot showing expression of Mucdhl induced by doxycycline in representative HCT116 clones. Multiple bands reflect glycosylation of Mucdhl. **(B)** Control or Mucdhl-expressing clones were seeded at low density and cultivated in absence (-) or presence of doxycycline (+) in the medium. Results of a representative experiment performed in triplicates are shown. Number of colonies (-/+ SD) counted in the absence of doxycycline for each clone was set at 100%. The inset contains bright-field microscopy images showing examples of colonies formed by a Mucdhl M clone. **(C)** Same as in (B) with clones seeded in soft agar. **(D)** Mucdhl M inducible cells were grown for 7 days without doxycycline and sub-sequently treated or not by doxycycline. Bright-field microscopy images show examples of a colony formed after 11 and 21 days of culture. Phase-contrast microscopy images show all the colonies (paraffin-embedded / H&E stained) after 21 days of culture. Original magnification 200X, except for paraffin-embedded colonies at 50X.

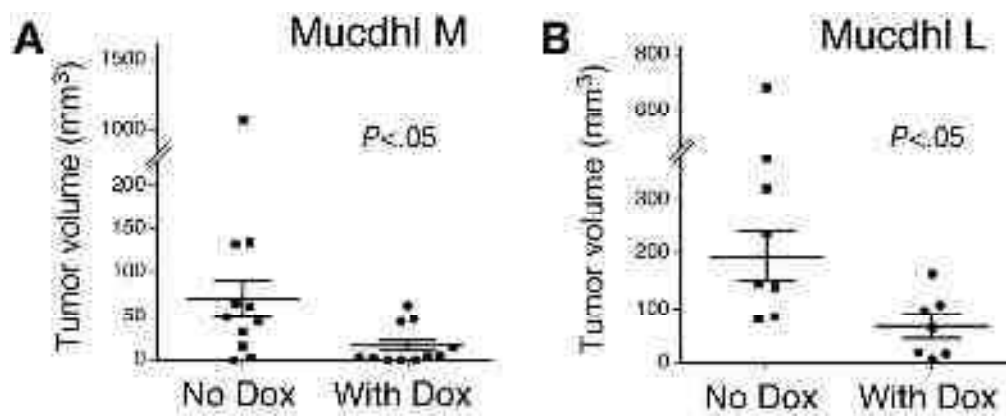


Figure 6: Mucdhl can reduce tumor formation of colon cancer cells

(A) Volumes (-/+ SEM) of sub-cutaneous tumors formed upon injection of control (- dox, squares) or expressing Mucdhl M (+ dox, dots) HCT116 stable cells in nude mice. **(B)** as in (A) with a Mucdhl L clone.

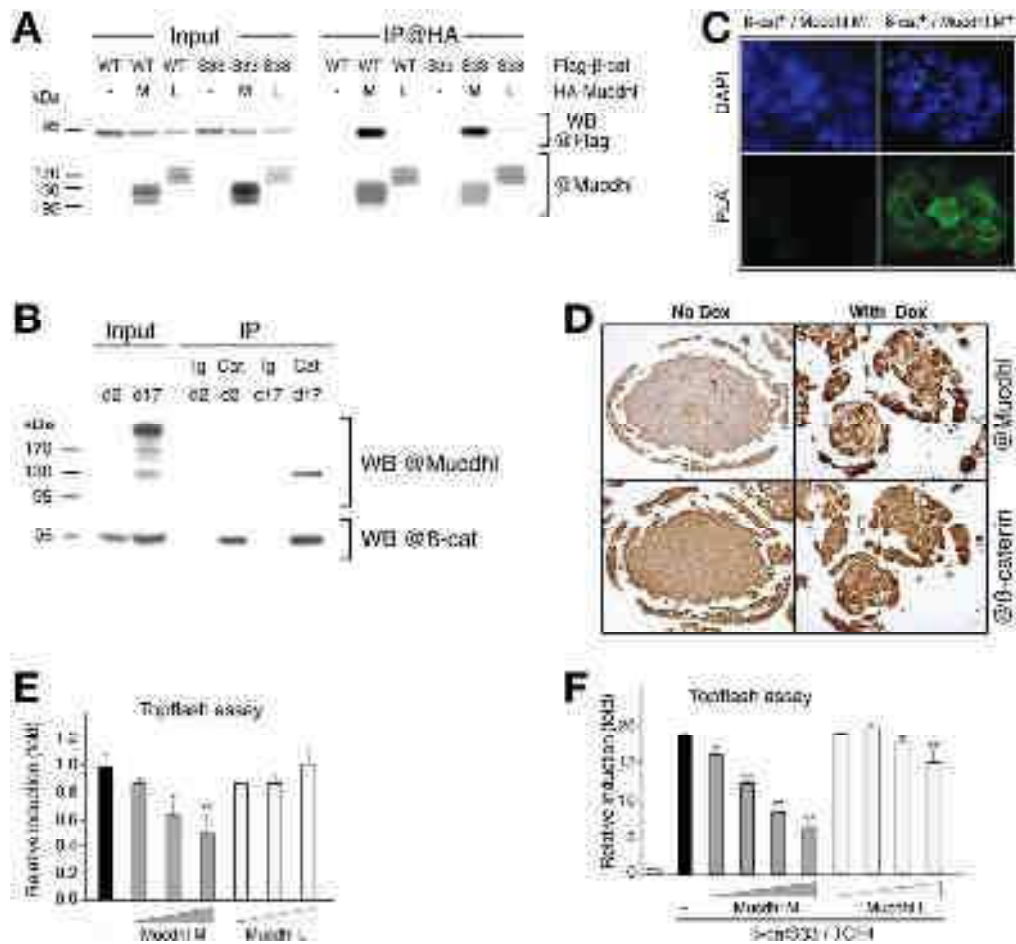
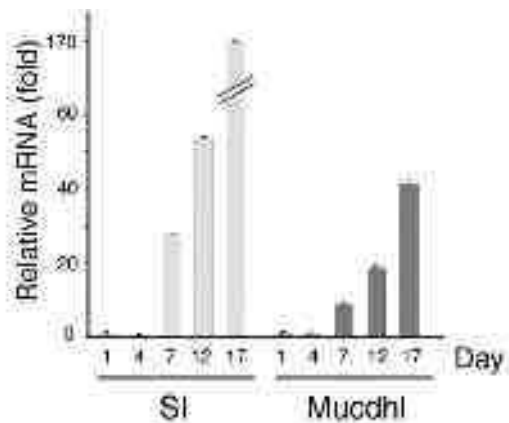


Figure 7: Mucdhl M can interact with β -catenin and antagonize its transcriptional activity

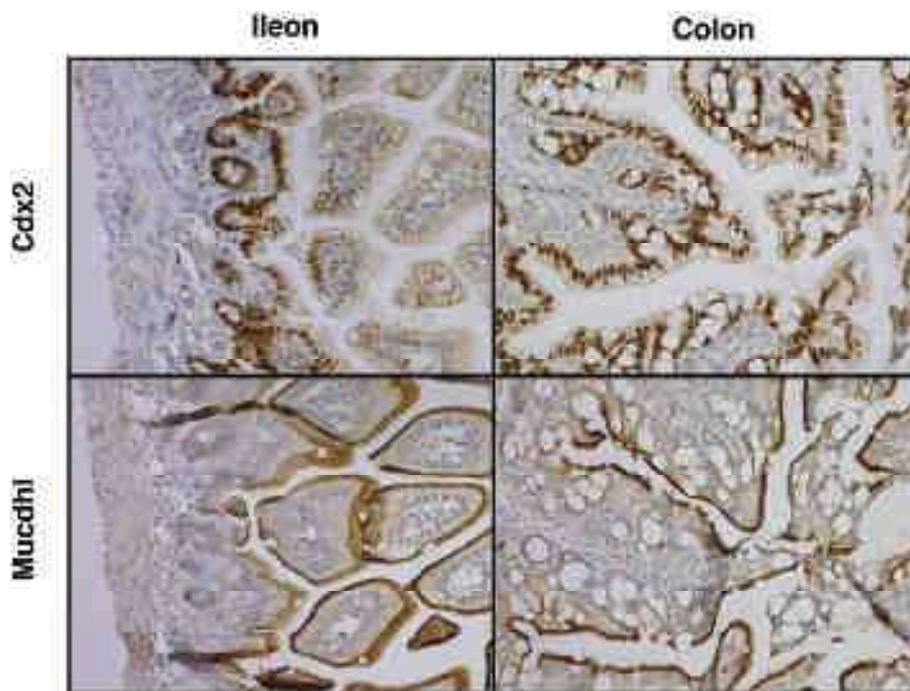
(A) HEK293 cells were transfected with Flag- β -catenin and HA-Mucdhl plasmids before immunoprecipitation (IP) with an anti-HA antibody. Proteins in bulk lysates (inputs, 1%) and immunoprecipitates were separated by SDS-PAGE and detected by Western blot. **(B)** Extracts from Caco2/TC7 grown for 2 or 17 days were incubated with IgG or an anti- β -catenin (cat) antibody for IP before analysis by Western blot. **(C)** HEK293 cells were transfected to overexpress β -catenin alone or with Flag-tagged Mucdhl M before in situ PLA using anti-Flag and anti- β -catenin antibodies. Green fluorescence corresponds to the PLA positive signal and indicates that the two molecules belong to the same protein complex; blue fluorescence corresponds to nuclei (DAPI). Original magnification: 400X. **(D)** Stable Mucdhl M-expressing HCT116 cells were grown in soft agar for 21 days with treatment (-/+ doxycycline) starting at day 7. Colonies were paraffin-embedded, sectioned and immunostained. Original magnification: 200X. **(E)** SW480 cells were transfected with increasing doses of Mucdhl-expressing plasmid or empty plasmid (-), the Topflash reporter plasmid and a promoter-less renilla reporter plasmid before luminescence measurement. Relative promoter activity (-/+ SD, normalized with renilla values) of the Topflash reporter in the absence of Mucdhl was set at 1. **(F)** As in (E) with HEK293 cells being transfected with β -catenin S33 and TCF4-expressing plasmids or empty plasmids (-).

Supplementary Figures



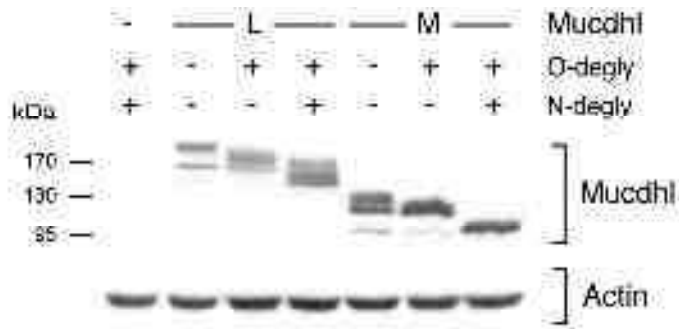
Supplementary Figure 1: Mucdhl expression correlates with intestinal differentiation

Caco-2/TC7 cells were grown for the indicated time to induce spontaneous differentiation before RNA extraction and RT-qPCR analysis of Mucdhl and SI (differentiation positive control) expression levels. Results are presented as folds of induction for each gene, with the level (-/+ SD, normalized with TBP expression) in cells grown for one day set at 1. Mucdhl transcripts are increasing after \approx 7 days of culture, when cells start to differentiate.



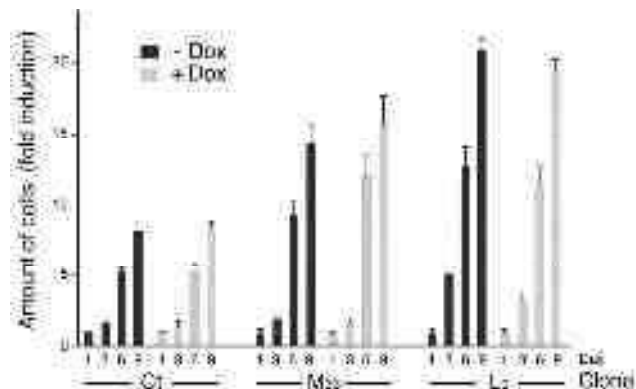
Supplementary Figure 2: Expression of Mucdhl in the murine intestine

Cdx2 and Mucdhl Antibodies were used on paraffin-embedded sections of P11 murine ileon or colon. Original magnification 200x.



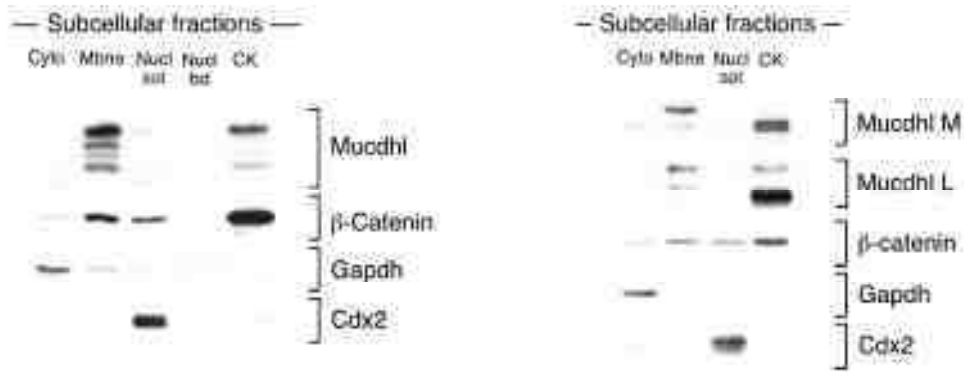
Supplementary Figure 3: Glycosylation of Mucdhl

HEK293 were transiently transfected with a Mucdhl-expressing plasmid (M or L) or the corresponding empty plasmid (-) and lysates were incubated with various deglycosylases to remove O-glycosylation (O-degly +), N-glycosylation (N-degly +) or both (O-degly +, N-degly +). Proteins were separated by SDS-PAGE and detected by Western blot. Treatment of extracts with deglycosylases reduces the number of bands detected by the anti-Mucdhl antibody (accumulation of faster migrating forms), strongly suggesting that Mucdhl isoforms are N and O-glycosylated. The blot was also incubated with an anti-Actin antibody to check protein loading and transfer.



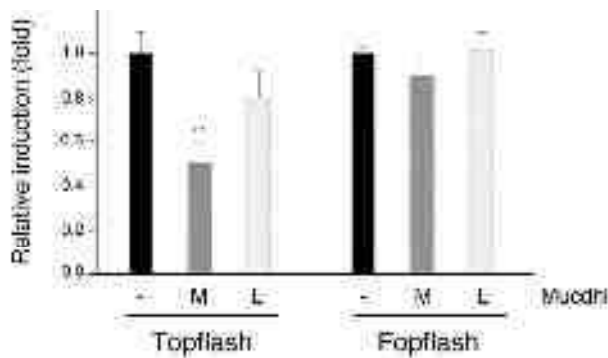
Supplementary Figure 4: Impact of Mucdhl on cell growth in standard culture conditions

Control or Mucdhl-expressing clones (M33 and L9) were grown at standard density in absence (- Dox) or presence (+ Dox) of doxycycline in the culture medium. The amount of cells at the indicated time point (1 to 9 days) was evaluated using a colorimetric MTS test. Results of a typical experiment with measurements performed on 6 wells are shown. For each clone, the OD (-/+ SD) measured on day 1 with/without doxycycline was set at 1 to facilitate comparison. Results indicate that expression of Mucdhl M or Mucdhl L has no impact on cell growth in standard culture conditions.



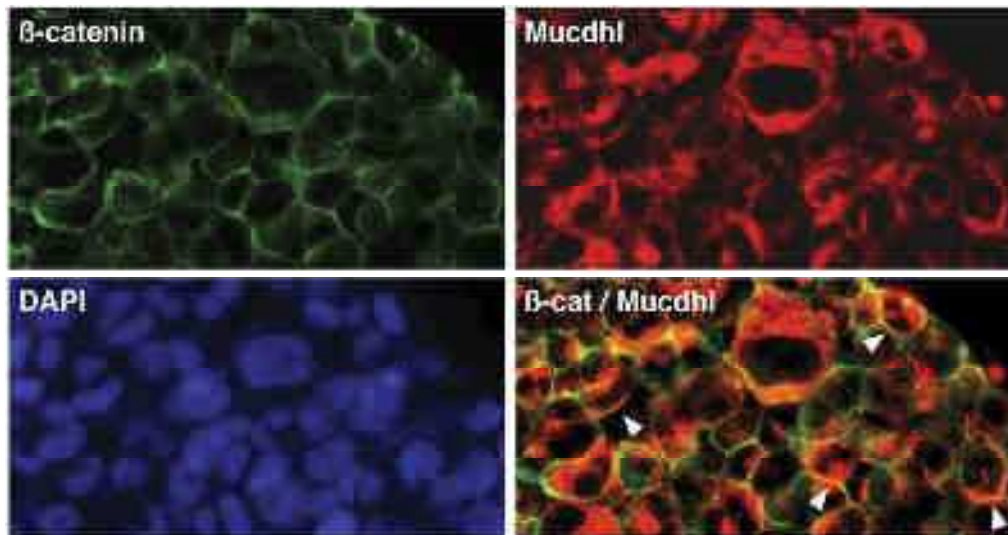
Supplementary Figure 5: Subcellular localization of Mucdhl

(A) Caco2/TC7 cells were grown for 2 weeks before lysis, sub-cellular fractionation, separation of the proteins by SDS-PAGE and analysis by Western blot with the indicated antibodies (Cyto: cytoplasmic fraction; Mbne: membrane fraction; Nucl sol: soluble nuclear fraction; Nucl bd: chromatin-bound nuclear fraction; CK: cytoskeletal fraction). Mucdhl is mainly detected in the membrane and cytoskeletal fractions. β -catenin is detected in the same fractions, but also in the nuclear and cytoplasmic fractions. Cdx2 (only nuclear) and Gapdh (mostly cytoplasmic) were detected to check the efficiency of the fractionation procedure. **(B)** As in (A) with proteins (Cdx2, Mucdhl M or Mucdhl L) overexpressed in HCT116. Mucdhl M and Mucdhl L are mainly detected in the membrane and cytoskeletal fractions, with the different bands exhibiting preferential localization: this suggests that glycosylated forms accumulate at the membrane.



Supplementary Figure 6: Specificity of the pTopflash reporter inhibition

SW480 cells were transfected with Mucdhl-expressing plasmids (M or L) or empty plasmid (-), the pTopflash or the control pFopflash reporter plasmid and a promoter-less renilla reporter plasmid before luminescence measurement. Relative promoter activity (-/+SD, normalized with renilla values) of each firefly luciferase reporter in the absence of Mucdhl was set at 1. Expression of Mucdhl M inhibits the activity of the pTopflash reporter but not of the control pFopflash reporter, showing that the TCF4 binding sites on the reporter are required for its effect.



Supplementary Figure 7: Colocalization of Mucdhl and β -catenin

Stable Mucdhl-M expressing HCT116 cells were grown in soft agar for 21 days with doxycycline treatment starting at day 7. Colonies were fixed, paraffin-embedded, sectioned, immunostained with a mix of anti- β -catenin (green fluorescence) and anti-Mucdhl (red fluorescence) antibodies. Nuclei were counterstained with DAPI (blue fluorescence). Co-localization of both proteins was assessed by the presence of a fluorescence merging (yellow fluorescence) resulting by the overlap of green and red fluorescence signals. Images (original magnification 400x) of a representative area show that β -catenin is mostly membranous, whereas Mucdhl M is both membranous and cytoplasmic. The merged fluorescence indicates plasma membrane localization (examples shown with white arrowheads). Co-localization was also confirmed using a plug-in of the ImageJ software.

Discussion and Perspectives

Mucdhl exhibits a restricted pattern of expression, with the intestinal epithelium being a major spot of synthesis (Moulton et al., 2004). Given that members of the cadherin superfamily mediate critical interactions for organ morphogenesis and homeostasis (Gumbiner, 2005; Halbleib and Nelson, 2006), we explored the mechanisms regulating the expression of Mucdhl and the function of its two main isoforms in intestinal cells. The results obtained indicate that Mucdhl is associated with some of the key players of intestinal biology and tumorigenesis, i.e. Cdx2 and β -catenin, and thus its loss in gut pathologies might be of functional significance.

1. Mucdhl is a target gene of Cdx2

1.1 Cdx2 regulates Mucdhl *in vitro*

The starting point of this study was that Mucdhl was differentially expressed in samples from two types of *in vitro* experimental models having in common the modification of Cdx2 expression. Very few genes had their expression modified in the two models considered, probably because they involved different cell lines and changes of numerous genes besides Cdx2. Given that LI-cadherin, a previously identified Cdx2 target (Hinoi et al., 2002), was also amongst these common genes, we hypothesized that Mucdhl might be a target of Cdx2. Indeed, we gathered compelling evidence that Cdx2 directly controls the transcription of Mucdhl in intestinal cell lines and identified the two Cdx2-binding sites on the Mucdhl promoter that are required for its activation. The most proximal of these sites exhibited the highest activity in luciferase assays, which was consistent with the fact that it corresponded more to the sequence of the canonical Cdx2 binding site (Margalit et al., 1993). Mutation of the more distal Cdx2 binding site, which slightly diverges from the consensus sequence, had a weaker impact on promoter activation.

1.2 *In vivo* confirmation of Mucdhl regulation by Cdx2

Importantly, the expression pattern of Mucdhl in the intestine was compatible with a regulation by Cdx2. Indeed, Mucdhl is predominantly expressed in differentiated cells of the intestine. In line with this, Mucdhl expression increases together with Cdx2 in Caco2/TC7 as these cells differentiate in cell culture. Furthermore, its expression is drastically reduced in pathological conditions involving decreased Cdx2 expression (foregut-type heteroplasia of Cdx2^{+/-} mice and colon tumors). Of note, microarray-based analysis of ileum samples from E18.5 embryos in which Cdx2 was homozygously inactivated in the endoderm also predicted a strong decrease of Mucdhl expression (Gao et al., 2009). Similarly, in pathological conditions showing ectopic expression of Cdx2 in non-intestinal

tissues (Barrett's esophagus, ovarian cancer), *Mucdhl* expression was up-regulated (Helm et al., 2005; Heinzlmann-Schwarz et al., 2006).

1.3 Other transcription factors co-activate *Mucdhl* expression in the intestinal epithelium

Altogether, these results strongly demonstrate that *Cdx2* is regulating the intestinal expression of *Mucdhl*. However, it is likely that other transcription factors are also involved in this process, as *Mucdhl* expression is detected later than *Cdx2* in the developing gut and as the level of stimulation of *Mucdhl* expression by *Cdx2* depends on the cell line considered (Figure 1E). A potential candidate for *Mucdhl* regulation is *HNF4α*, as in intestine-specific *HNF4α* Knockout mice *Mucdhl* expression was reduced even though *Cdx2* expression was not affected (Garrison et al., 2006). Furthermore, it was shown that *HNF4α* bound to the *Mucdhl* promoter in the murine intestine, which was later confirmed by a ChIP on Chip analysis (Boyd et al., 2009; Garrison et al., 2006).

Nevertheless, our results clearly show that *Mucdhl* is a direct target gene of *Cdx2* and *Cdx2* is the first transcriptional regulator of *Mucdhl* identified to date.

2. *Mucdhl* function in intestinal cells

We started to investigate the function and mode of action of *Mucdhl* in intestinal cells. In contrast to *Cdx2*, ectopic expression of *Mucdhl* L or M had no effect on cell growth in standard conditions of culture (Gross et al., 2008). However, when cells grew in clusters, expression of *Mucdhl* M drastically reduced the formation and maintenance of the colonies. Although clonal bias cannot be excluded, overexpression of *Mucdhl* L seemed less efficient, suggesting that the two isoforms are not functionally equivalent. The mechanism of colony inhibition remains unclear. A direct effect on proliferation induced by the low amount of cells (i.g. lack of secreted growth factor) is possible. *Mucdhl* may also impact on cell survival in stringent conditions as even established colonies collapsed upon *Mucdhl* M expression. Of note, cleaved Caspase 3 (indicative of apoptosis) was not detected in these conditions. In cells that detach from the substrate or that grow in extreme low density, changes in signaling pathways have to occur to permit cell survival in the new conditions (Guadamillas et al., 2011). *Mucdhl* might interfere with these pathways, a possibility that might explain why no effect on cell proliferation was seen in normal culture conditions.

Another possibility is that alteration of intercellular adhesion potentially induced by overexpression of *Mucdhl* leads to cell death as previously reported for other molecules (Ruoslahti and Reed, 1994). This hypothesis is supported by: i) the observation that *Mucdhl* M-expressing colonies appeared irregularly shaped and were formed of oddly organized cells, ii) the fact that

ectopic expression of rat Mucdhl L in fibroblasts induced their aggregation (Goldberg et al., 2000). However, in our hands, Mucdhl had no effect in aggregation assays (not shown). Finally the unusual predominant apical location of Mucdhl *in vivo* prompted Goldberg and colleagues to suggest that glycosylated Mucdhl may polarize the cells and thus overexpression may disorient them within the colony (Goldberg et al., 2000). In the intestinal epithelium and in cell culture Mucdhl is predominantly expressed on the apical cell surface (Goldberg et al., 2002). However, in cells grown in 3D culture conditions, the presence of Mucdhl on the whole cell surface might permit its interaction with other partners and induce signaling that triggers cell death.

Future studies combining cellular and animal models will clarify the process induced by Mucdhl in intestinal cells, but it is to be noted that the possible mechanisms are matching Cdx2's cellular activities (Gao and Kaestner, 2010; Gross et al., 2008; Suh and Traber, 1996; Mallo et al., 1998).

3. Mode of action of Mucdhl

3.1 Interaction with β -catenin

Concerning the mode of action of Mucdhl, we clearly showed that Mucdhl M can directly interact with β -catenin as suggested by colocalization of the 2 molecules at the membrane of some cells (**Supplementary Figure 7**) (Parenti et al., 2010). This colocalization was first detected by others in kidney cells (Goldberg et al., 2000), then more clearly in intestinal cells (Parenti et al., 2010), and finally confirmed in the gut (Losi et al., 2011). In addition, we found that overexpression of Mucdhl M reduced the transcriptional activity of β -catenin. The most obvious scenario is that the binding of Mucdhl M prevents the nuclear translocation of β -catenin, although such trapping was only observed in Mucdhl M-expressing colonies grown in soft agar. However, given the complexity of β -catenin shuttling and regulation, others faced similar difficulties in detecting E-cadherin-induced membrane retention of β -catenin (Gottardi and Gumbiner, 2001). Nevertheless, recent data indicated a correlation between Mucdhl expression and membrane retention of β -catenin in colon tumors (Losi et al., 2011).

Other possibilities of β -catenin inhibition include the modification of β -catenin interaction with partners and/or of its phosphorylation state that can modulate its nuclear localization, transcriptional activity or stability. Indeed, even though the PLA assay indicated that both proteins co-localized (distance < 30nm), it is still possible that Mucdhl and β -catenin interact indirectly via another partner. To better understand the nature of the Mucdhl / β -catenin interaction, it would be helpful to know which protein domains are implicated using purified recombinant proteins.

Meanwhile, several important aspects can already be taken into consideration: i) both the expression and activity of Cdx2 have previously been connected to β -catenin, indicating a strong interdependence between these important actors of the intestinal physiopathology; ii) Mucdhl is predominantly expressed in differentiated cells of the intestine, revealing an inverse correlation with proliferation and transcriptional activity of β -catenin (Kim et al., 2007); iii) the interaction with β -catenin essentially concerned Mucdhl M, indicating for the first time that both isoforms are not functionally equivalent.

This last aspect is in line with the lower efficiency of Mucdhl L in reducing colony formation but was unexpected as β -catenin usually interacts with the intracellular domain of cadherin molecules. The presence of the mucin domain may modify the conformation of Mucdhl L or the binding to other molecules, thus preventing interaction with β -catenin.

3.2 Implication of Mucdhl in other pathways

Given that Mucdhl L exerts some inhibitory effect on colony formation in soft agar, it is very likely that the activity of Mucdhl isoforms is also mediated by β -catenin-independent mechanisms. The SH3 or PDZ-binding motifs and the predicted phosphorylation sites in the intracellular part of Mucdhl may participate to its signaling activity. Mucdhl is potentially interacting with the cytoskeleton, as most cadherins and also membrane-bound mucins bind directly or indirectly to the cytoskeleton (Angst et al., 2001; Hollingsworth and Swanson, 2004). Accordingly, an important proportion of Mucdhl was found in the cytoskeleton-associated fraction in subcellular fractionation experiments (**Supplementary Figure 5**).

To identify pathways in which one or both splicing isoforms of Mucdhl are implicated different strategies can be engaged. One possibility would be to compare the transcriptomes of cells expressing or not Mucdhl by DNA microarray or RNA-Seq (high-throughput sequencing). The activity of signaling pathways may also be compared in these cells, using for instance phospho-specific antibodies. Another approach would be to identify proteins interacting with Mucdhl M or Mucdhl L by a yeast two-hybrid screen or by performing mass spectrometry analysis of proteins co-immunoprecipitating / co-purifying with Mucdhl.

4. Effect of Mucdhl on tumor development

4.1 Mucdhl inhibits tumor growth and its expression is down-regulated in colon cancer

Both Mucdhl splicing isoforms inhibited tumor growth in nude mice. Consistently with this result, in the majority of the human tumors that we analyzed by immunohistochemistry and RT-qPCR, Mucdhl was down-regulated compared to the normal intestinal tissue. Our current results indicate that expression of both Mucdhl splicing isoforms is reduced at the same level in human tumors, but the size of our cohort is too small to make a definitive conclusion. A study by Losi et al confirmed our data, as they found that Mucdhl expression was reduced in 71% of the analyzed tumors. Importantly, the presence of Mucdhl was inversely correlated with the frequency of proliferating cells and nuclear β -catenin staining, but not with the tumor stage (Losi et al., 2011). Similarly, in DNA microarray screens analyzing human colon adenomas (Sabates-Bellver et al., 2007) or tumors of MIN mice (a model for Familial adenomatous polyposis (Martinez et al., 2005)), Mucdhl was down-regulated compared to normal mucosa.

These interesting results justify studying Mucdhl expression in a larger cohort of colon patients, using for instance TMAs (tissue microarrays), which will consolidate the data and also permit to detect a potential association of Mucdhl expression with tumor stage / grade, the tumor genotype and clinical outcome. This type of study could reveal if Mucdhl could be used as a molecular marker in colon cancer diagnosis and treatment decision in colon cancer.

If future results confirm the loss of expression and the anti-oncogenic potential of Mucdhl, it will be important to understand by which mechanism Mucdhl expression is lost in colon tumors. Beside chromosomal alteration of the Mucdhl gene, possible mechanisms are promoter methylation, or its down-regulation by cancer-associated signaling pathways. Surprisingly, in the tumor samples that we have analyzed by RT-qPCR, Cdx2 expression was not reduced (data not shown). This may be explained by the fact that Cdx2 loss of expression in colon tumors is heterogeneous and thus the total level of Cdx2 transcripts might not be significantly altered. In addition, it should be kept in mind that Cdx2 is also regulated at the post-transcriptional level: detection of Cdx2 by RT-qPCR, or even by immunohistochemistry, does not automatically implicate that Cdx2 is transcriptionally active. Indeed, in the last years, several studies showed that Cdx2 stability / activity can be regulated by phosphorylations or the expression of a dominant negative splicing isoform lacking the transactivation domain (Houde et al., 2001; Gross et al., 2005; Lemieux et al., 2011; Modica et al., 2009). However, the observation that the loss of Mucdhl expression might take place without concomitant down-regulation of Cdx2 expression, as already found in colon adenomas (Sabates-

Bellver et al., 2007), supports our assumption that Mucdhl is regulated by a combination of transcription factors. For instance, the down-regulation of HNF4 α in colon tumors might contribute to the loss of Mucdhl expression (Saandi et al, in press).

The loss of expression of Mucdhl in human colon cancer may only be a consequence of the tumor development. However, the fact that Mucdhl inhibits growth of colon cancer cells *in vitro* and *in vivo* suggests that its loss may contribute to tumorigenesis. To determine the influence of Mucdhl on tumor initiation and progression, Mucdhl^{-/-} mice, which are viable and fertile (Tang et al., 2010), could be used. It would be interesting to see if these mice develop colon tumors spontaneously or if they show enhanced tumorigenesis upon treatment with the colon carcinogen AOM or crossing with APC^{+/ Δ 14} mice. The tumor characteristics (volume, proliferation rate, differentiation, aggressiveness...) of Mucdhl^{-/-} mice could be compared with those of their wild-type littermates. The impact of Mucdhl loss of expression would be especially interesting in the context of in APC^{+/ Δ 14} mice, as Mucdhl inhibits β -catenin activity and its loss correlates with nuclear β -catenin in colon cancer samples (Losi et al., 2011).

5. Further perspectives for the study of Mucdhl

5.1 Mucdhl and IBDs

Inflammatory bowel diseases (IBDs) include Crohn's disease and ulcerative colitis, which are chronic inflammatory conditions. Interestingly, Mucdhl expression is down-regulated in DNA microarrays performed on samples from IBD patients (Losi et al., 2011). Moreover, Mucdhl expression is highly induced upon 5-ASA (5-aminosalicylic acid, also known as mesalazine) treatment in Caco2 colon cancer cells (Parenti et al., 2010). 5-ASA is an anti-inflammatory agent used in the symptomatic treatment of IBDs (Velayos et al., 2005). 5-ASA reduces cell proliferation, inhibits β -catenin signaling and promotes apoptosis (Parenti et al., 2010), but its exact mode of action remains elusive. Because Mucdhl can also impact cell proliferation and inhibit β -catenin, it may be a potential effector of the 5-ASA activity in IBDs.

The potential implication of Mucdhl in IBD development and treatment response also links Mucdhl to CRC, as patients with IBDs have an increased risk of developing intestinal tumors (Ballinger and Anggiansah, 2007) and as 5-ASA might exert a chemopreventive activity against CRC (Velayos et al., 2005), even though this effect is still debated (Bernstein et al., 2011).

To assess the implication of Mucdhl in the development of IBD and the response of patients to treatments, the expression of Mucdhl will have to be examined by immunohistochemistry or RT-qPCR

in samples of IBD patients (normally appearing colonic mucosa – inflamed, non-dysplastic mucosa – low or high grade dysplasia, colitis-associated cancer – impact of 5-ASA treatment). In parallel, experimental animal models of intestinal inflammation could be used. One possibility is to treat *Mucdhl*^{-/-} mice with DSS, a polymer that is added to the drinking water and which damages the intestinal epithelium. This leads to exposure to microbes / soluble factors in the intestinal lumen that will induce a chronic inflammation (Wirtz and Neurath, 2007). Another interesting model for intestinal inflammation is provided by *IL-10*^{-/-} mice, which spontaneously develop chronic enterocolitis (Wirtz and Neurath, 2007). Comparison of compound *Mucdhl*^{-/-} *IL-10*^{-/-} mice with single Knockout mice may determinate if *Mucdhl* expression affects the development of the disease, its progression, severity and response to anti-inflammatory treatments.

5.2 Function and physiological role of *Mucdhl*

Although *Mucdhl* is strongly expressed in the intestinal epithelium, we do not have yet many clues about its actual physiological role in the intestinal epithelium.

Mucdhl could be implicated in the integrity of the intestinal barrier function, as altered expression of both mucin and cadherin can change the epithelial permeability (Schneider et al., 2010). A defective intestinal barrier will allow recurrent exposure of the intestinal epithelium to the content of the lumen (pathogenic bacteria, toxic molecules...), thereby leading to chronic inflammation and increasing the risk of cancer.

Intriguingly, in the small intestine and colon, *Mucdhl* is expressed on the lateral borders of the cells and in the cytoplasm, but the strongest staining is detected at the apical side of the cells. At first sight, this may seem surprising, as the main role of cadherins is to mediate cell-cell adhesion. However, it has become evident in the last years that in addition to cell adhesion, cadherins also impact on proliferation, polarity, sub-cellular trafficking or cytoskeletal organization (see IV)3 page 58). Ectopic expression of *Mucdhl* could enhance intercellular adhesion of rat fibroblasts (Goldberg et al., 2000), but we failed to see any differences in the aggregation of intestinal cells (data not shown), perhaps because of the presence of other cadherins in these cells. Nevertheless, it is possible that *Mucdhl* does not play an important role in cell adhesion in the intestine, but may be involved in other cell functions, as it has already been shown for several other non-classical cadherins. Indeed, many other cadherins were reported to be localized at the apical cell side of cells, such as T-cadherin (Koller and Ranscht, 1996), *Pcdh24* (Protocadherin LKC) (Krahn et al., 2010), Fat and Dachous (Ishiuchi et al., 2009). Fat and Dachous are atypical cadherins that mediate PCP (planar cell polarity) (Karpowicz et al., 2010; Ishiuchi et al., 2009) and *Pcdh24* was suggested to be implicated in the polarization of the

intestinal epithelium (Krahn et al., 2010). The fact that Mucdhl and FAT ectodomains are more similar to each other than to any other cadherin (Goldberg et al., 2000) and the apical localization of Mucdhl suggest that it is also implicated in the polarization of epithelial cells. In this context, a possible role for Mucdhl would be as a sensor for cell polarization. Mucdhl may also interact with other cell surface molecules and/or secreted mucins and modulate cell behavior by outside-in signaling (Hollingsworth and Swanson, 2004). Thus, the exact sub-cellular localization of Mucdhl, its potential implication in junction complexes like adherens junction or in specialized membrane domains, as lipid rafts, which have been shown to serve as platforms for signaling pathways, should be assessed as it will give valuable information to understand the function of Mucdhl.

Finally, an aspect that also requires further investigations is the glycosylation status of Mucdhl (**Supplementary Figure 3**), as it is evident that the correct glycosylation of proteins is essential to their function and it is clear now that aberrant glycosylation of proteins is implicated in cancer (Byrd and Bresalier, 2004). Changes in glycosylation could be an additional level of regulation of Mucdhl activity and function and this is especially true for the long isoform containing the mucin domain, as mucins are heavily-glycosylated. In this context, Goldberg et al showed that the glycosylation of Mucdhl is important for its function in kidney development (Goldberg et al., 2000).

In vitro experiments that aim at determining the consequences of Mucdhl expression and at identifying the proteins interacting with Mucdhl (see above) will provide some additional clues about its role in intestinal cells. In addition, the mice invalidated for Mucdhl will be of great use to study the physiological role of Mucdhl in the intestinal epithelium, as well as in other organs expressing Mucdhl, such as the kidney, the liver, and the lung (Goldberg et al., 2000, 2002; Moulton et al., 2004) (Lü et al., 2004;). The consequences of Mucdhl absence on the morphology (Ex: length, thickness, crypt-villus axis) of the intestinal epithelium, its composition (Ex: proportion of proliferative cells, differentiated cell types) and functionality (Ex: permeability, stool production) could be examined. In particular, electron microscopy imaging of the microvilli structure on the apical cell surface would be interesting, given that Mucdhl is primarily expressed at the brush border formed by the microvilli and that Mucdhl potentially interacts with the cytoskeleton, a critical element of the microvilli. If these analyses indicate that Mucdhl^{-/-} mice are significantly different from their wild-type littermates, further functional (Ex: metabolic cages) and molecular (Ex: RNA-Seq) exploration could be envisaged and provide supplementary information concerning the physiological role of Mucdhl.

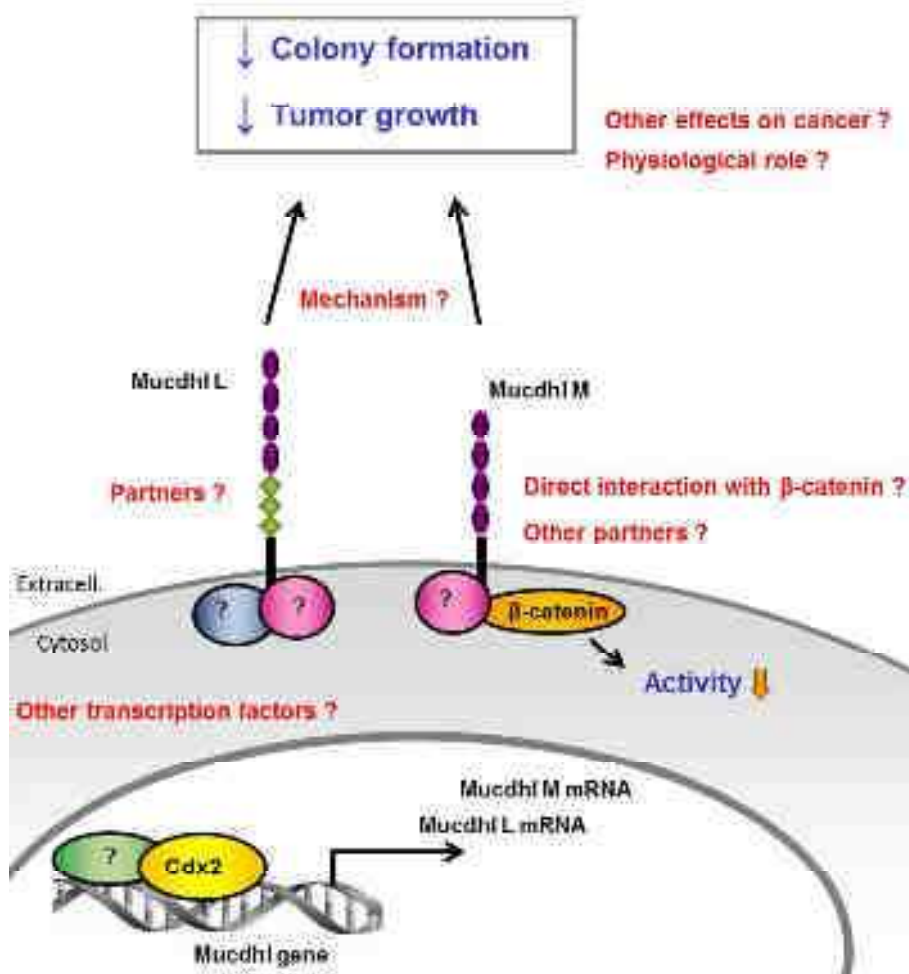


Figure 8: Principal results and remaining questions concerning Mucdhl

6. Conclusion

In summary, we identified Mucdhl as a new Cdx2 target gene and started to explore its mode of action (Figure 8). The role of Mucdhl in the intestinal epithelium is not yet known but its expression is altered in several pathologies of the gut. Indeed, we found that Mucdhl expression was reduced in experimentally induced murine colon tumors as well as in most samples of a small collection of human colon cancers. Similar results were obtained in parallel with a bigger cohort of patients (Losi et al., 2011). This loss of Mucdhl might have functional consequences given that forced expression of Mucdhl opposed tumor formation in nude mice. For instance, the loss of Mucdhl might contribute to the enhanced nuclear localization of β-catenin that correlates with the decrease of Cdx2 in invasive colon cancer cells (Brabletz et al., 2004). Altogether, these important observations warrant further investigation of Mucdhl in the gut.

GENERAL CONCLUSION

General conclusion

My thesis work unraveled new functions and target genes of Cdx2, that permit to better apprehend its physiological function and its action as a tumor suppressor in the intestine. Indeed, the identification and characterization of two new target genes of Cdx2, the cadherin Mucdhl and the RhoGEF Vav3, confirmed its implication into the epithelial phenotype of the intestine, especially in the processes leading to cell differentiation and tissue integrity (cell adhesion and rigidity of the epithelium). The inhibitory effect of Mucdhl on the growth of colon cancer cells and on beta-catenin activity are also in line with its role as a tumor suppressor. Similarly, the unexpected impact of Cdx2 on the cell cytoskeleton that we have discovered may be critical for the structural organization of the intestinal epithelium, but also mediate the inhibitory effect of Cdx2 on cancer cell migration. Taken together, our data strongly suggest that Cdx2 opposes tumor growth and metastasis by enforcing the epithelial phenotype.

Beside the study of the mode of action of Cdx2, I also developed a new cell model to study tumor heterogeneity through a Cdx2 reporter cell line. After its validation, this cell line will be a precious tool to study *in vivo* the molecular mechanisms underlying tumor heterogeneity and its consequences, as it seems that the tumor cell plasticity reflected by the tumor heterogeneity is a prerequisite for metastasis formation. The most important applications of this cell line would be to i) identify and validate new molecular markers for diagnosis/prognosis of colon tumors; ii) identify genes or signaling pathways that might be new drug targets to inhibit the tumor plasticity. A combination of these new drugs with classical cytotoxic agents might complete the surgical removal of the tumor and improve the life expectancy of colon cancer patients.

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PUBLICATIONS AND COMMUNICATIONS

PUBLICATIONS

Publication #1

Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I. (2012). Cdx2 controls expression of the protocadherin Mucdhl, an inhibitor of growth and β -Catenin activity in colon cancer cells. *Gastroenterology*. Apr;142(4):875-885.e3. (Impact Factor =12)

Publication #2

Hinkel I.*, Platet N.*, Richert L., Senger B., Vautier D., Vannier M., Gaiddon C., Voegel J.C., Freund J.N. & Gross I. The homeotic tumor suppressor Cdx2 opposes elasticity of colon cancer cells through reorganization of the actin cytoskeleton and enhanced cell-cell contacts.
In préparation. * co-authors

COMMUNICATIONS

Posters:

“Mucdhl, a new target gene of the tumor suppressor Cdx2” par Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I.

- Cancérologie du Grand-Est, Strasbourg, novembre 2010
- FASEB Summer Research Conference, Steamboat Springs (Etats-Unis), août 2011
- Models and Mechanisms of Cancer, Cold Spring Harbor (Etats-Unis), août 2012

Oral Communications:

“Mupcdh et Muc13, deux nouveaux gènes cibles du suppresseur de tumeurs coliques Cdx2” par Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I.

- CECED XXVIII, Paris, mars 2010

“Mucdhl, un nouveau gène cible du suppresseur de tumeurs Cdx2 inhibe la formation de colonies et l'activité de la β -caténine” par Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I.

- Journée des doctorants de la faculté de médecine, Strasbourg, novembre 2011
- CECED XXX, Grenoble, mars 2012

“Mucdhl, a target gene of the tumor suppressor Cdx2, inhibits colony formation and β -catenin activity” par Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I.

- EUCC meeting, Freiburg, mai 2012

ANNEX 1

Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I. (2012). Cdx2 controls expression of the protocadherin Mucdhl, an inhibitor of growth and β -Catenin activity in colon cancer cells. *Gastroenterology*. Apr;142(4):875-885.e3. (Impact Factor =12)

Cdx2 Controls Expression of the Protocadherin Mucdhl, an Inhibitor of Growth and β -Catenin Activity in Colon Cancer Cells

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BACKGROUND & AIMS: The intestine-specific homeobox transcription factor Cdx2 is an important determinant of intestinal identity in the embryonic endoderm and regulates the balance between proliferation and differentiation in the adult intestinal epithelium. Human colon tumors often lose Cdx2 expression, and heterozygous inactivation of Cdx2 in mice increases colon tumorigenesis. We sought to identify Cdx2 target genes to determine how it contributes to intestinal homeostasis.

METHODS: We used expression profiling analysis to identify genes that are regulated by Cdx2 in colon cancer cells lines. Regulation and function of a potential target gene were further investigated using various cell assays.

RESULTS: In colon cancer cell lines, Cdx2 directly regulated the transcription of the gene that encodes the protocadherin Mucdhl. Mucdhl localized to the apex of differentiated cells in the intestinal epithelium, and its expression was reduced in most human colon tumors. Overexpression of Mucdhl inhibited low-density proliferation of colon cancer cells and reduced tumor formation in nude mice. One isoform of Mucdhl interacted with β -catenin and inhibited its transcriptional activity. **CONCLUSIONS: The transcription factor Cdx2 activates expression of the protocadherin Mucdhl, which interacts with β -catenin and regulates activities of intestinal cells. Loss of Cdx2 expression in colon cancer cells might reduce expression of Mucdhl and thereby lead to tumor formation.**

Keywords: Tumor Suppressor; *cdhr5*; Colorectal Cancer; CRC.

Despite its complex architecture, the intestinal epithelium represents the most vigorously renewing adult tissue in mammals.¹ Thus, dynamic temporal and spatial coordination of various cellular processes is not only required during fetal development of the gut but also throughout adult life to maintain homeostasis.

Alterations of the signaling pathways that ensure controlled renewal of the gut are frequently found in gut pathologies, notably colorectal cancer,² which corresponds to the second cause of cancer-related death in the Western world.³ This is well illustrated by the prominent loss of function of APC, which in particular regulates the protein levels of β -catenin: in addition to its adhesive function at the membrane, stabilized β -catenin relays in the nucleus the activity of the Wnt pathway, which has a

central role in the control of proliferation, cell stemness, differentiation, and migration in the gut.^{4,5}

Another molecule required for the homeostasis of the gut and whose expression is frequently reduced in colon tumors⁶ is the homeodomain transcription factor Cdx2. Cdx2 specifies cell fate or axial position at different developmental stages,⁷ but a series of recent studies confirmed that Cdx2 is a major player in both the developing and the mature intestinal epithelium. Cdx2 expression in the embryo per se is detected around embryonic day (E) 8.5 and becomes progressively restricted to the intestinal epithelium at the adult stage.⁸ Endoderm-specific Cdx2 ablation from E9.5 onward results in colon agenesis, with the ectopic activation of a foregut/esophageal differentiation program reflecting an anterior homeotic transformation.⁹ Intestine-specific Cdx2 ablation around E14 is also lethal and leads to abnormal morphogenesis of the small intestine, with shortened villi and poorly differentiated, nonpolarized enterocytes.¹⁰ Strikingly, even intestine-specific loss of Cdx2 at the adult stage led to chronic diarrhea and death, with small intestine dilation and immature enterocytes, indicating that Cdx2 is required to maintain gut homeostasis throughout life.¹¹ Accordingly, reduction of Cdx2 expression promotes tumor progression in animal models of colon cancer,^{12,13} whereas forced expression of Cdx2 in colon cancer cells decreases metastasis in nude mice.¹⁴

Altogether, these data correlate quite well with experiments performed in vitro. Indeed, forced expression of Cdx2 in intestinal cell lines enhances intestinal differentiation^{15,16} and adhesion¹⁷ while decreasing proliferation.^{14,18} On the opposite, silencing of Cdx2 disrupts apicobasal polarity¹⁰ and enhances motility.¹⁴ On the molecular level, a certain number of genes regulated by Cdx2 have been identified, namely intestine-specific genes involved in the digestive function.⁸ As direct targets mediating the other functions of Cdx2 remain more elusive, we took advantage of microarray-based studies performed with intestinal cells showing modification of Cdx2 expression. One of the genes whose expression paralleled the one of Cdx2 was *Cdhr5*, also called Mucin and cadherin like

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; PLA, proximal ligation assay; qPCR, quantitative polymerase chain reaction; RT, reverse transcription.

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(Mucdhl). This gene encodes several splicing isoforms of a protocadherin mostly expressed in the kidney, liver, and gut.^{19–21} Protocadherins represent a group of less conserved members of the Cadherin superfamily that are membranous glycoproteins mediating Ca²⁺-dependent cell-to-cell adhesion. All Mucdhl isoforms exhibit 4 cadherin extracellular motifs but differ by the presence of several mucin-like tandem repeats located downstream of the cadherin motifs, a transmembrane domain, and an intracellular domain containing many potential protein-binding motifs.^{20,21} In this study, we investigated the regulation of Mucdhl expression in intestinal cells and initiated a comparative analysis of the function and mode of action of Mucdhl isoforms.

Materials and Methods

Small Interfering RNA

Stealth/small interfering RNA (siRNA) duplex oligoribonucleotides were from Invitrogen (Carlsbad, CA): CDX2HSS141544 (siRNA@Cdx2 544), CDX2HSS141546 (siRNA@Cdx2 546), and medium GC Duplex number 2 as negative control.

Mucdhl-Expressing Cell Lines

The T-REx System (Invitrogen) was used to establish control and Mucdhl-inducible cell lines. First, HCT116TR cells, which constitutively express the tetracycline repressor, were established by transfection of HCT116 cells with pcDNA6/TR and blasticidin selection. Clone 7 of HCT116TR was transfected with pcDNA4/TO, pcDNA4/TO-DHLM/Flag or pcDNA4/TO-DHLL/HA and submitted to Zeocin selection. Several independent stable clones were obtained that expressed Flag-tagged Mucdhl M or HA-tagged Mucdhl L. These clones were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, Blasticidin (4 µg/mL; Invitrogen), Zeocin (100 µg/mL; Invitrogen), and antibiotics. Induction of expression was performed by adding doxycycline (1 µg/mL; Sigma, St. Louis, MO) to the medium.

Wound Healing and Grafting

Wound healing assay with Caco2/TC7 cells and grafting of HT29 cells were previously described.^{14,22} Subcutaneous injections of HCT116 Mucdhl stable cells (1.10⁶ cells/each side) in female nu/nu mice (Elevage Janvier, France) were performed similarly, with half of the mice receiving doxycycline (400 µg/mL) in their drinking water.^{14,22} Mice were killed (clone M, 8 weeks; clone L, 4 weeks), and tumors measured.

Transfections

Plasmid transfections were performed with Lipofectamine 2000 (Invitrogen) or Jetprime (Polyplus Transfection, Illkirch, France), except for Luciferase assays in which JetPEI (Polyplus Transfection) was used. siRNA duplexes were transfected with Lipofectamine RNAiMAX (Invitrogen).

RNA Extraction and Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated using TRI Reagent (MRC, Cincinnati, OH). Semiquantitative reverse-transcription polymerase chain reactions (RT-PCR) were performed as described¹⁴ with the following primer pairs: hMucdhl L (forward: CTC CCA CCA ACC AAC CAC; reverse: CAT ATC CAC CAC CGA GAA GC),

hMucdhl M (forward: TGG AGG GAG AGG TTG TGC T; reverse: GGC CGC CAC CTG TGG AGG), hCdx2 (forward: AAG CCG AGC TAG CCG CCA CGC TGG; reverse: TGA GAG CCA GGT CTG TAG GTC TAT) and as internal control hTBP (forward: CGT GTG AAG ATA ACC CAA G; reverse: ATT GGA CTA AAG ATA GGG A).

Luciferase Assays

Luciferase assays were performed with the Dual-Luciferase Reporter Assay (Promega, Madison, WI) as described.¹⁴ Data presented correspond to a representative experiment performed in triplicate.

In Situ Proximal Ligation Assay

Cells seeded in 8-well CultureSlides (BD Falcon, Mountain View, CA) were transfected and treated overnight with MG132 (10 µmol/L; Sigma) before fixation (Paraformaldehyde 4%, 15 minutes) and permeabilization (Triton X-100 0.5% in phosphate-buffered saline, 30 minutes). Slides were processed for in situ proximal ligation assay (PLA) with the Duolink II Detection Reagents Green, Duolink II PLA probe anti-Mouse PLUS, and Duolink II PLA probe anti-Rabbit Minus (Olink Bioscience, Uppsala, Sweden) as recommended. Primary antibodies were rabbit anti-β-catenin (clone 6B3, 1/150; Cell Signaling Technology, Danvers, MA) and mouse anti-Flag (clone M2, 1/500; Sigma). To check the specificity, control experiments omitting one of the primary antibodies were performed. Imaging was based on optical sectioning using the ApoTome system (Zeiss, Oberkochen, Germany).

Colony Formation and Soft Agar Assays

For colony formation assays, 250 cells in 1 mL of medium were added to 1 mL of medium without or with doxycycline (2 µg/mL) per well of a 6-well plate (3 replicates) and allowed to grow for 14 days before fixation (70% ethanol), staining with Crystal violet (0.05% in phosphate-buffered saline), and counting of the colonies.

For soft agar assays, cells were mixed with Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 0.4% low melting point agarose (Promega); 1.5 mL of this solution (5000 cells) were added to each well of a 6-well plate previously coated with 1.5 mL of 0.6% low melting point agarose (3 replicates). After 30 minutes, 1 mL of growth medium without or with doxycycline (1 µg/mL) was added per well. Medium was changed every 2–3 days. After 3 weeks, colonies were fixed, stained, and counted.

Statistical Analysis

P* < .01 and *P* < .001 given by a 1-way analysis of variance test followed by a Newman-Keuls test. Additional materials and methods are presented in Supplementary Materials and Methods.

Results

Expression of Mucdhl Depends on the Presence of Cdx2 in Intestinal Cells

We previously reported similar changes in messenger RNA expression and/or protein distribution during in vitro wound healing and tumor dissemination in colon cancer for several molecules. In particular, β-catenin accumulated in the nucleus of migrating cells at the wound edge or at the invasive front of tumors, whereas Cdx2 expression was down-regulated in both situations.¹⁴ To

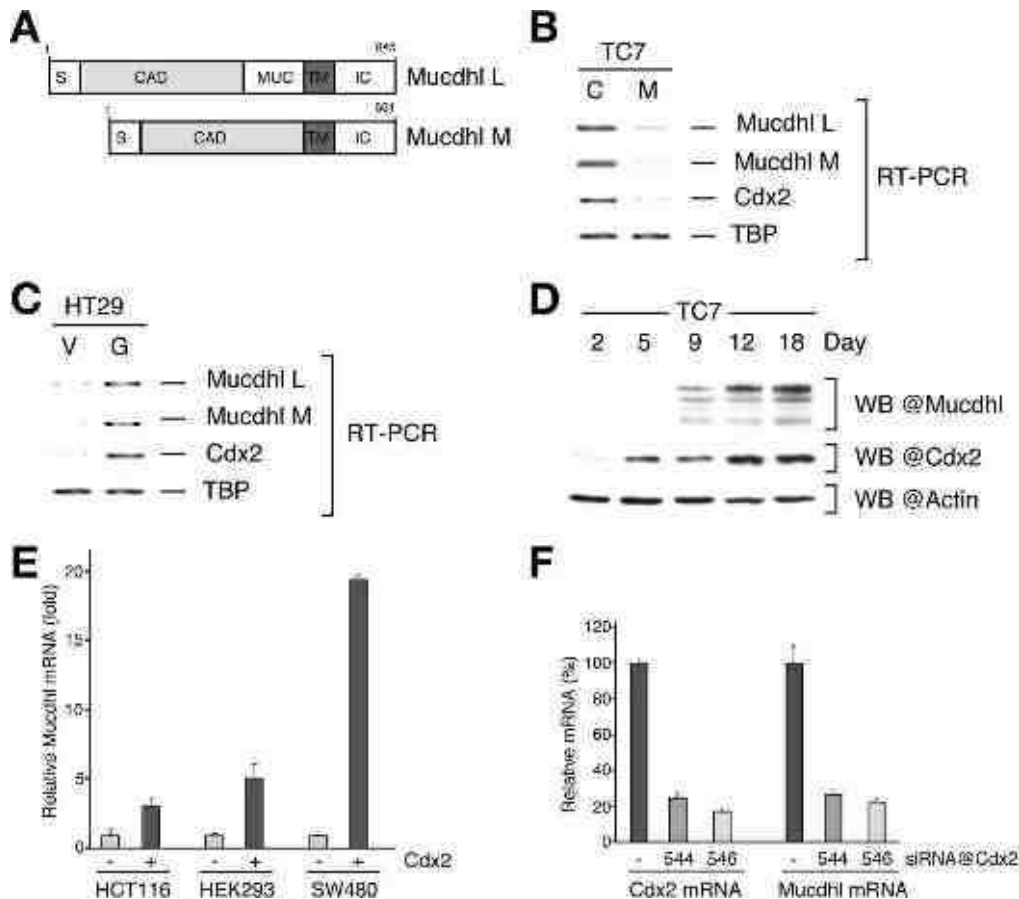


Figure 1. Mucdhl expression depends on Cdx2. (A) Schematic representation of human Mucdhl L and M (CAD, cadherin motifs; IC, intracellular domain; MUC, mucin-like tandem repeats; S, signal peptide; TM, transmembrane domain). (B) A monolayer of Caco-2/TC7 cells was wounded, and, 48 hours later, cells located at the wound edge (M) or in the confluent monolayer (C) were separately collected for RNA extraction and RT-PCR with TBP as internal control. (C) HT29 were cultivated in vitro (V) or subcutaneously grafted (G) before RNA extraction and RT-PCR. (D) Caco-2/TC7 cells were grown for the indicated time before Western blot analysis. The multiple bands detected by the anti-Mucdhl antibody reflect as reported the presence of several isoforms and glycosylation.^{19–21} (E) Cdx2 was overexpressed (+) by transfection before RNA extraction and RT-qPCR. Results are presented as fold of induction for each cell line, with the level of Mucdhl expression (\pm standard deviation, normalized with TBP expression) in cells without Cdx2 overexpression set at 1. (F) SW480 cells were transfected with control (–) or Cdx2-specific small interfering RNA before RNA extraction and RT-qPCR. Results are presented as percentage of expression with the level of Cdx2 or Mucdhl expression (\pm standard deviation, normalized with TBP) in the control sample set at 100%.

obtain a global view of the transcriptional events occurring, we performed exploratory gene-profiling analysis on Caco-2/TC7 cells located either at the wound edge or in the confluent monolayer. One of the genes whose expression was predicted to be down-regulated in migrating cells was Mucdhl. Strikingly, the expression of Mucdhl was also modified in another microarray analysis in which transcriptomes from HT29 colon cancer cells grown in vitro or grafted in nude mice (an experimental setup that revealed the influence of the microenvironment on Cdx2 expression) were compared.²² As in both cases Mucdhl and Cdx2 expressions were similarly modified, we hypothesized that Mucdhl might be a transcriptional target of Cdx2. First, we verified the data of the microarrays by RT-PCR for the 2 major isoforms of Mucdhl detected in intestinal cells: Mucdhl M and Mucdhl L (Figure 1A–C). Second, we observed that, similarly to Cdx2, expression of Mucdhl rises during spontaneous differentiation of Caco-2/TC7 intestinal cells grown at confluence (Figure 1D and Supplementary Figure 1).

Next, we performed direct gain and loss of function experiments to confirm that Mucdhl expression depends on Cdx2. When Cdx2 was overexpressed in colon (HCT116, SW480) or renal (HEK293) cell lines, Mucdhl expression determined by quantitative RT-PCR (RT-qPCR) was increased (Figure 1E). RT-PCR using isoform-specific primers confirmed that the augmentation affected both isoforms (not shown). When Cdx2 expression was silenced with 2 different siRNA in SW480 cells, Mucdhl expression was concomitantly decreased (Figure 1F). Thus, Cdx2 not only enhances but is also required for Mucdhl expression in intestinal cells.

Mucdhl Is a Transcriptional Target of Cdx2

Examination of the 3-kilobase DNA region upstream of the coding sequence of Mucdhl revealed the presence of potential Cdx2-binding sites. To determine whether Cdx2 is able to bind to the Mucdhl promoter, we performed chromatin immunoprecipitation (ChIP) experiments in HCT116 cells, which express low levels of en-

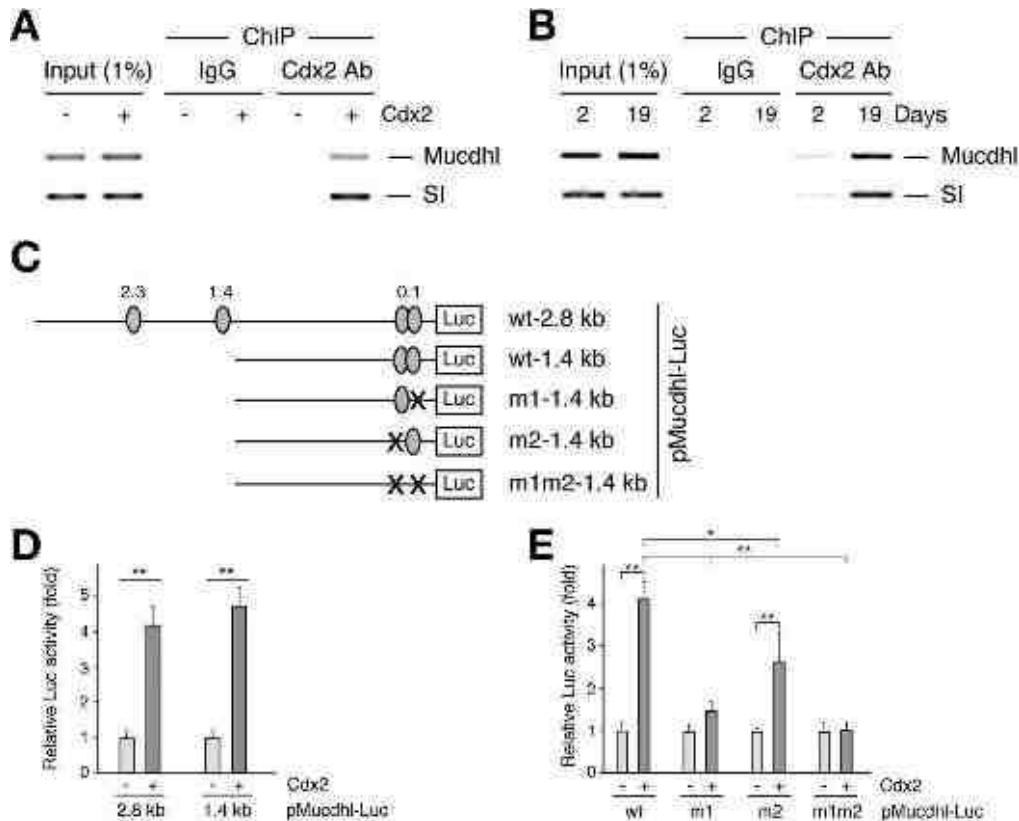


Figure 2. Cdx2 regulates the transcription of Mucdhl. (A) Chromatin of HCT116 cells expressing Cdx2 (+) or not (–) was subjected to immunoprecipitation (ChIP) with an anti-Cdx2 antibody or control IgG before PCR detection of a Mucdhl or a sucrase-isomaltase (SI, positive control) promoter fragment. PCR was also performed with 1% of the bulk DNA (input) to ensure equivalence of the samples. (B) As in panel A with Caco2/TC7 grown for 2 or 19 days. (C) Schematic representation of the different Mucdhl reporter plasmids tested with the positions of the potential Cdx2-binding sites (intact, shaded ovals; mutated, X). (D) HCT116 were transfected with a Cdx2-expressing plasmid (+) or the corresponding empty plasmid (–), the indicated pMucdhl-Luc reporter plasmid, as well as a promoter-less renilla reporter plasmid (internal control) before lysis and luminescence measurement. Relative promoter activity (\pm standard deviation, normalized with renilla values) of each of the pMucdhl-Luc reporter in the absence of Cdx2 was set at 1. (E) As in panel D, using the indicated reporter plasmids. * $P < .01$ and ** $P < .001$.

ogenous Cdx2 and Mucdhl: upon overexpression of Cdx2, a DNA fragment corresponding to the Mucdhl promoter was amplified by PCR in Cdx2 immunoprecipitates (Figure 2A). Similarly, ChIP performed with differentiated Caco-2/TC7 extracts revealed the binding of endogenous Cdx2 to the Mucdhl promoter (Figure 2B).

To check whether Cdx2 regulates the transcription of Mucdhl, we generated 2 luciferase reporter plasmids, which differ by the size of the Mucdhl promoter region and thus the number of potential Cdx2-binding sites (Figure 2C). When transfected into HCT116 cells, the luciferase activity of these pMucdhl-Luc reporters was stimulated by Cdx2 (Figure 2D). Both reporters exhibited similar activation, suggesting that the required elements were contained within the 1.4-kilobase region upstream of the Mucdhl translation site. To determine whether any of the 2 Cdx2 binding sites present in this region was involved, we generated by site-directed mutagenesis additional pMucdhl-Luc reporters, harboring only 1 of the Cdx2-binding sites or none (Figure 2C): activation of the mutated pMucdhl-Luc reporters by Cdx2 was slightly reduced by the loss of the m2 site, severely decreased by the m1 site and abolished by the loss of the 2 sites (Figure 2E). Thus, both sites seem to be involved, with

the m1 playing a prominent role, consistent with the fact that its sequence is a better match of the consensus Cdx2-binding site. Similar results were obtained in SW480 cells (not shown). Altogether, this set of data indicates that Cdx2 directly regulates the transcription of Mucdhl by binding to its 2 proximal Cdx2 consensus sites.

Mucdhl Expression in the Intestinal Epithelium Is Reduced Upon Loss of Cdx2 and Tumorigenesis

In murine embryos, Mucdhl was detected by immunohistochemistry in the gut around E16.5, concomitant to intestinal cytodifferentiation (Figure 3A). In the mouse and human adult small intestine, Mucdhl was predominantly observed in the differentiated cells of the villi, in particular at the brush border (Figure 3B and Supplementary Figure 2). Expression was mainly detected at the plasma membrane, especially at the apex of the cells, and weakly in the cytoplasm. In the colon, Mucdhl exhibited an increasing gradient toward the surface of the epithelium, indicating that Mucdhl was more expressed in differentiated cells. In addition, consistent with the role of Cdx2 in Mucdhl expression, Mucdhl is lost in foregut-type

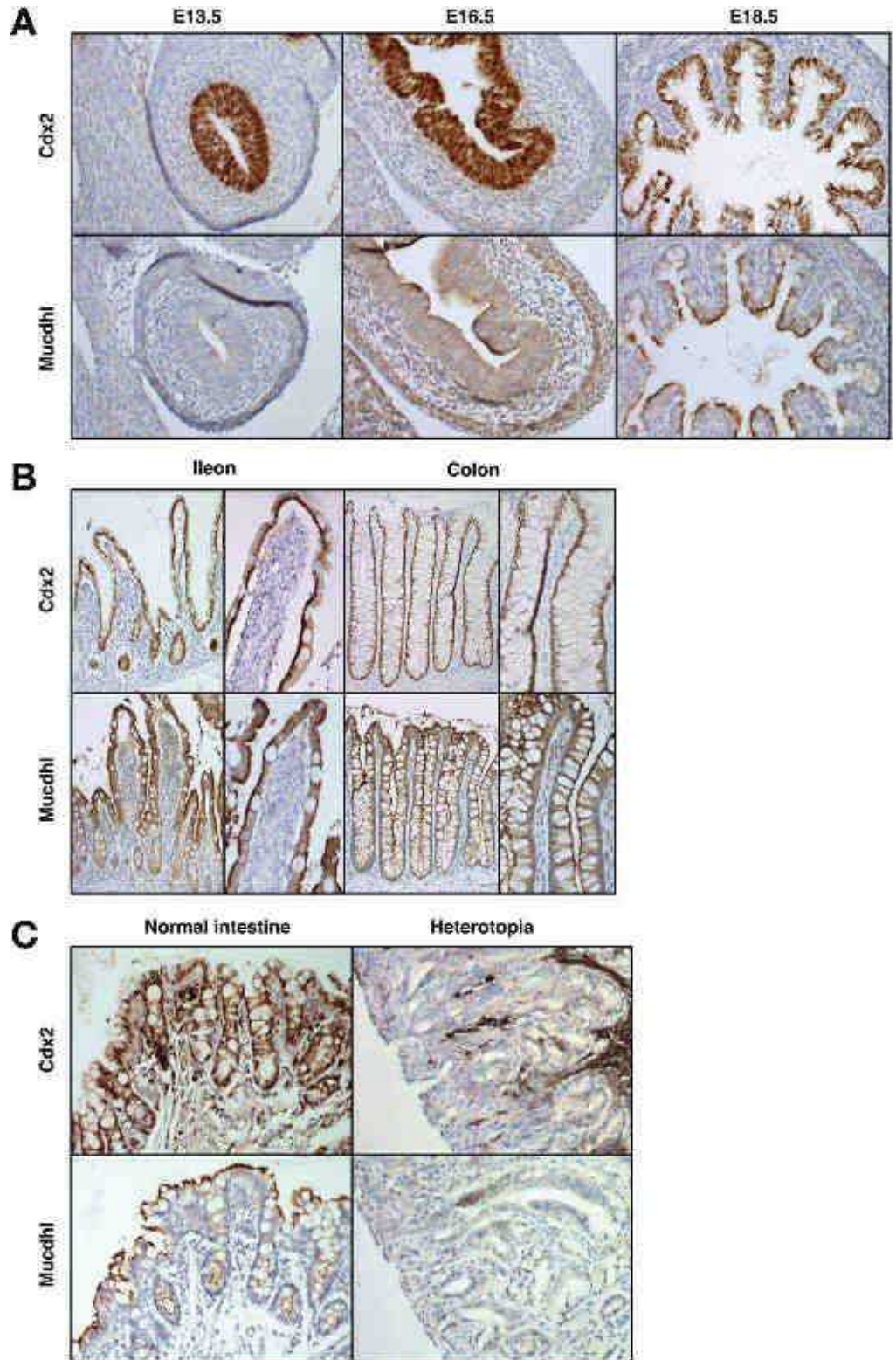


Figure 3. Expression of Mucdhl in wild-type and Cdx2^{+/-} intestines. Cdx2 and Mucdhl antibodies were used on paraffin-embedded sections. (A) Staged mouse embryos. (B) Adult human intestines. (C) Matching areas of normal intestinal epithelium and foregut-type heterotopia of a Cdx2^{+/-} mouse. Original magnification, ×200, except in panel B with ×100 (left panel) and ×200 (right panel).

heterotopia appearing in the pericecal lesions of Cdx2^{+/-} mice (Figure 3C) and totally lacking Cdx2 expression.²³

Finally, we examined Mucdhl expression in colon tumors. Mice treated with the azoxymethane carcinogen or tumor prone (APC^{L14/+})²⁴ exhibited colon tumors with reduced levels of Cdx2 and Mucdhl (Figure 4A). In human colon tumors, Mucdhl expression was also frequently lower than

in the normal epithelium, as shown by immunohistochemical staining and RT-qPCR (Figure 4B and C).

Mucdhl Can Reduce Colony and Tumor Formation of Colon Cancer Cells

To investigate the function of Mucdhl, we established stable cell lines expressing Mucdhl after doxycy-

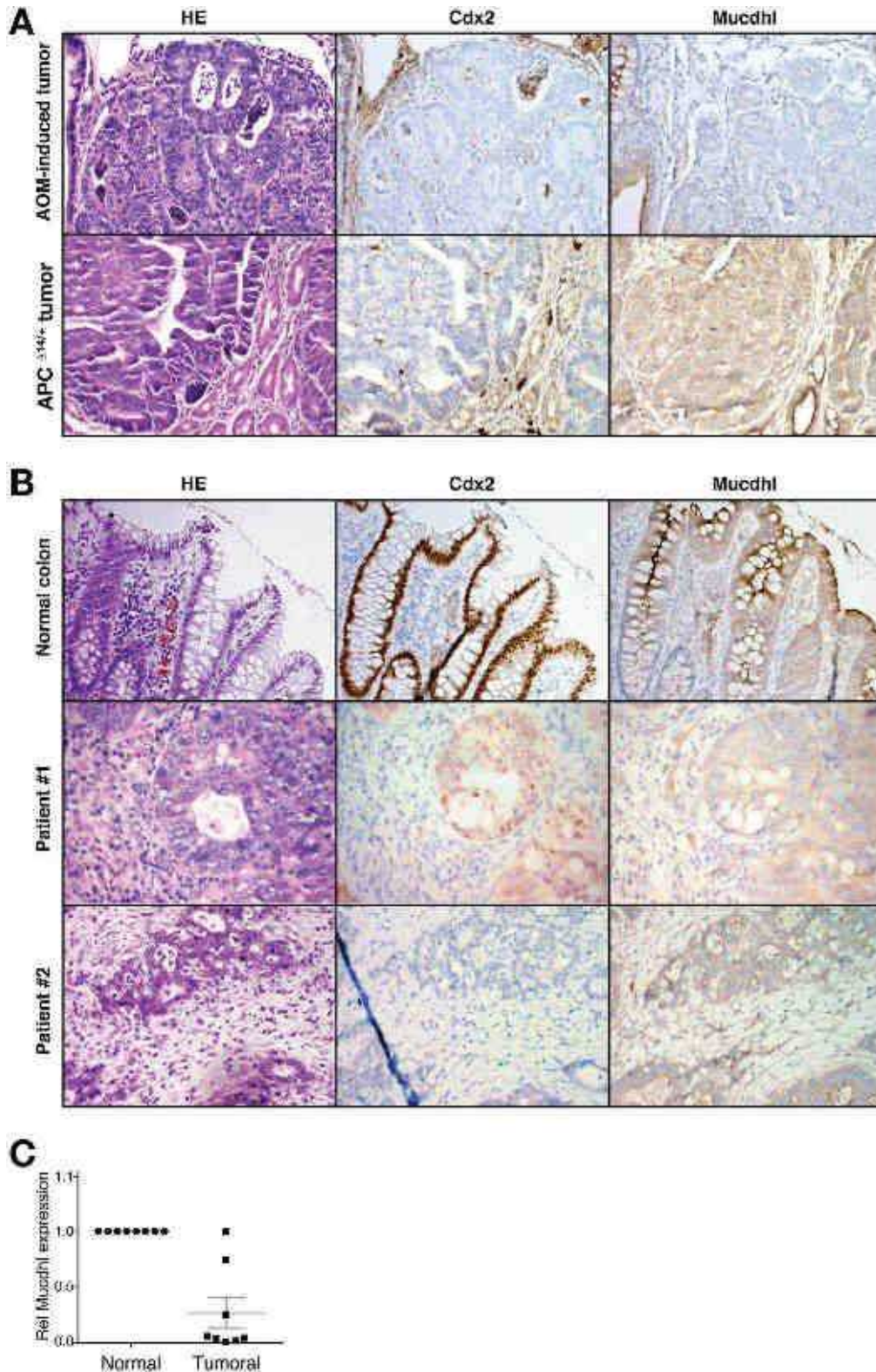


Figure 4. Mucdhl expression is reduced in colon tumors. (A) H&E staining and immunohistochemistry were performed on tumor tissue from the distal colon of azoxymethane (AOM)-treated mice or APC^{Δ14/+} mice. (B) H&E and immunohistochemistry were performed on normal colon and colon tumors from 2 patients (original magnification, ×200). (C) RT-qPCR were performed on RNA from matched normal (circles) or tumor (squares) colon tissues. Relative Mucdhl expression (±standard error mean, normalized with TBP) for each normal colon was set at 1.

cline treatment of HCT116 cells, which normally produce only trace amounts of endogenous Mucdhl (Figure 5A). For both isoforms, and as previously reported,¹⁹ multiple bands were detected by Western blot, which reflects post-translational modifications, mainly glycosylation (Supplementary Figure 3).

Growth of control and Mucdhl-expressing clones cultivated in standard conditions was equivalent (Supplementary Figure 4). In contrast, Mucdhl M clearly reduced the number of colonies in a colony formation assay (Figure 5B). The same effect was observed with Mucdhl L, although to a lower extent and with more variability between the clones

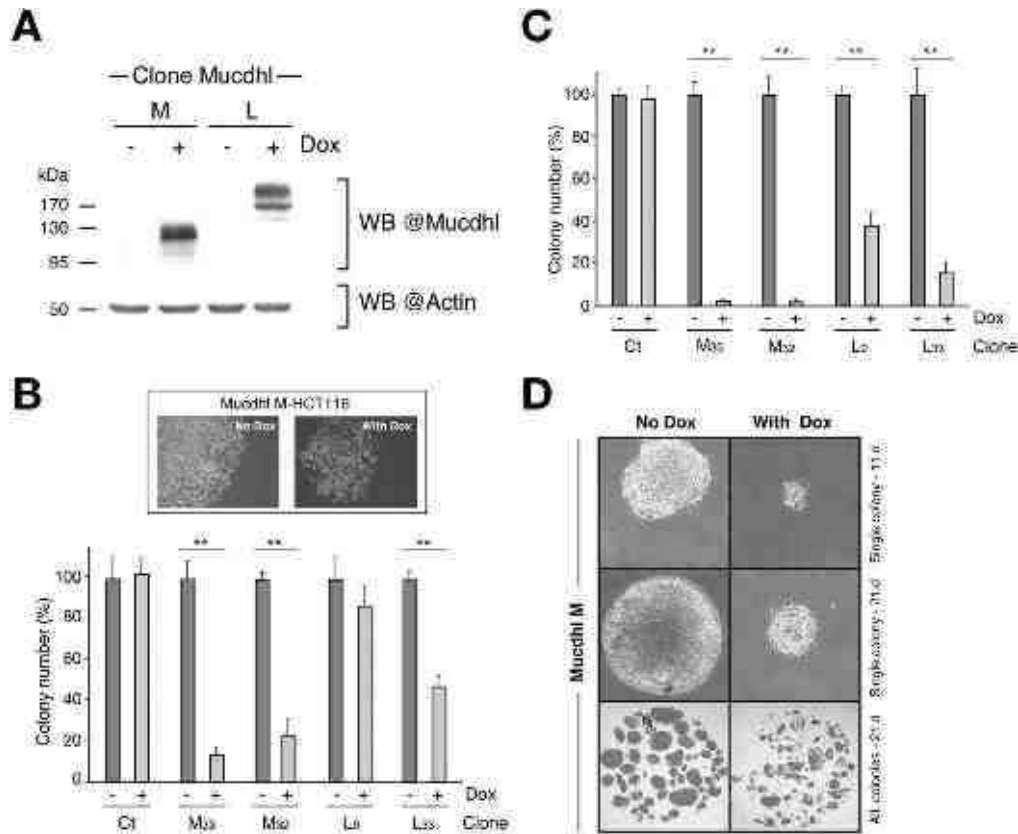


Figure 5. Mucdhl can reduce colony formation of colon cancer cells. (A) Western blot showing expression of Mucdhl induced by doxycycline in representative HCT116 clones. Multiple bands reflect glycosylation of Mucdhl. (B) Control or Mucdhl-expressing clones were seeded at low density and cultivated in absence (–) or presence of doxycycline (+) in the medium. Results of a representative experiment performed in triplicates are shown. Number of colonies (\pm standard deviation) counted in the absence of doxycycline for each clone was set at 100%. The inset contains phase-contrast microscopy images showing examples of colonies formed by a Mucdhl M clone. (C) Same as in panel B with clones seeded in soft agar. (D) Mucdhl M inducible cells were grown for 7 days without doxycycline and subsequently treated or not by doxycycline. Phase-contrast microscopy images show examples of a colony formed after 11 and 21 days of culture. Bright-field microscopy images show all the colonies (paraffin embedded/H&E stained) after 21 days of culture. Original magnification, $\times 200$, except for paraffin-embedded colonies at $\times 50$ (bottom panels). * $P < .01$ and ** $P < .001$.

but was not seen in control clones. In addition, the few colonies that formed in the presence of Mucdhl appeared smaller and disorganized (Figure 5B, inset). Colony formation was also examined in anchorage-independent conditions by plating the cells in soft agar or matrigel (not shown). Again, the number of colonies was reduced by Mucdhl, especially by Mucdhl M, which almost totally abolished

colony formation (Figure 5C). Even when cells were allowed to form colonies in soft agar for 7 days before addition of doxycycline, expression of Mucdhl still led to fewer, smaller, and more disorganized colonies (Figure 5D).

To determine the impact of Mucdhl on tumor formation in vivo, control and Mucdhl-expressing cells were injected under the skin of nude mice. Expression of either Mucdhl isoform led

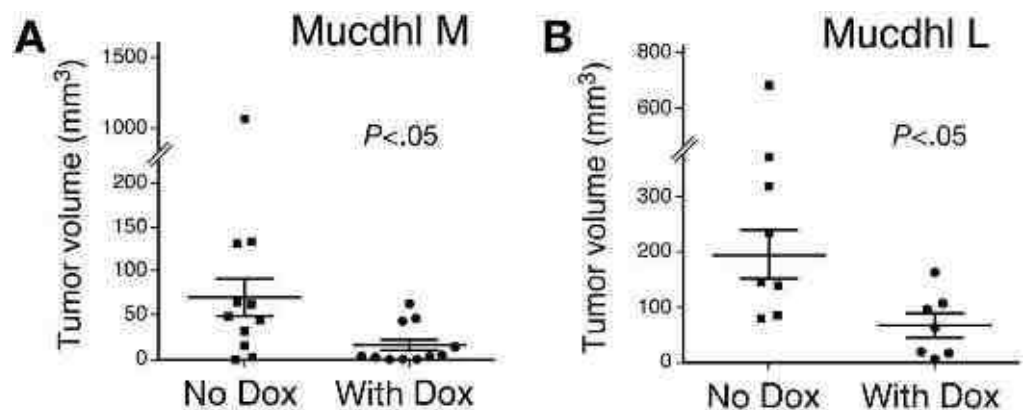


Figure 6. Mucdhl can reduce tumor formation of colon cancer cells. (A) Volumes (\pm standard error mean) of subcutaneous tumors formed upon injection of control (no doxycycline, squares) or expressing Mucdhl M (with doxycycline, circles) HCT116 stable cells in nude mice. (B) As in panel A with a Mucdhl L clone.

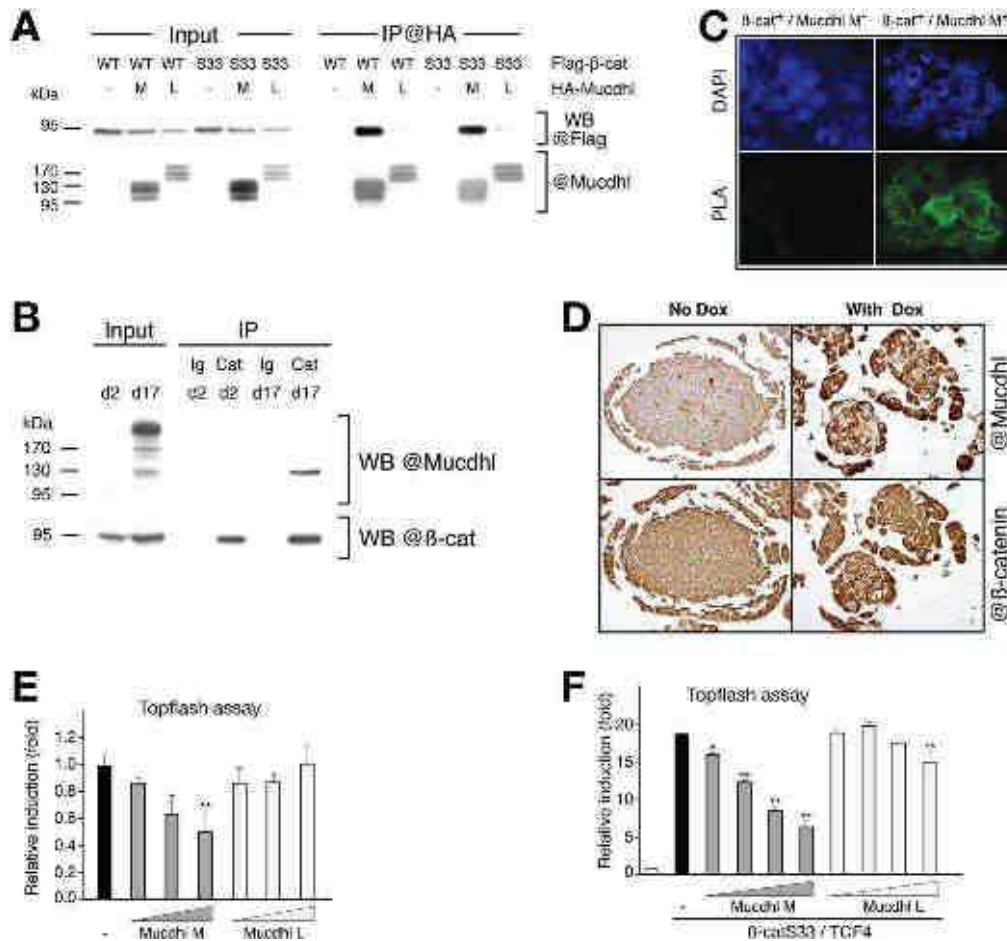


Figure 7. Mucdhl M can interact with β -catenin and antagonize its transcriptional activity. (A) HEK293 cells were transfected with Flag- β -catenin and HA-Mucdhl plasmids before immunoprecipitation (IP) with an anti-HA antibody. Proteins in bulk lysates (inputs, 1%) and immunoprecipitates were separated by SDS-PAGE and detected by Western blot. (B) Extracts from Caco2/TC7 grown for 2 or 17 days were incubated with IgG or an anti- β -catenin (cat) antibody for immunoprecipitation before analysis by Western blot. (C) HEK293 cells were transfected to overexpress β -catenin alone or with Flag-tagged Mucdhl M before in situ PLA using anti-Flag and anti- β -catenin antibodies. *Green fluorescence* corresponds to the PLA-positive signal and indicates that the 2 molecules belong to the same protein complex; *blue fluorescence* corresponds to nuclei (DAPI). Original magnification, $\times 400$. (D) Stable Mucdhl M-expressing HCT116 cells were grown in soft agar for 21 days with treatment (\pm doxycycline) starting at day 7. Colonies were paraffin-embedded, sectioned, and immunostained. Original magnification, $\times 200$. (E) SW480 cells were transfected with increasing doses of Mucdhl-expressing plasmid or empty plasmid (–), the Topflash reporter plasmid, and a promoter-less renilla reporter plasmid before luminescence measurement. Relative promoter activity (\pm standard deviation, normalized with renilla values) of the Topflash reporter in the absence of Mucdhl was set at 1. (F) As in panel E with HEK293 cells being transfected with β -catenin S33 and TCF4-expressing plasmids or empty plasmids. * $P < .01$ and ** $P < .001$.

to smaller subcutaneous tumors (Figure 6), indicating that both Mucdhl M and L can oppose tumor formation in vivo.

Mucdhl M Can Interact With β -Catenin and Reduce Its Transcriptional Activity

Because several members of the cadherin superfamily interact with β -catenin, we checked if this was the case for Mucdhl. Coimmunoprecipitation experiments were performed with extracts from HEK293 cells transfected with various combinations of expression vectors for β -catenin and the Mucdhl isoforms. As shown on Figure 7A, β -catenin (wild-type or S33Y stabilized mutant²⁵) coimmunoprecipitated with Mucdhl M. In contrast, β -catenin was barely detectable in immunoprecipitates of Mucdhl L. Similar results were obtained in HCT116 and SW480 cells (not shown). We also performed coimmunoprecipitation

experiments with Caco-2/TC7 cell extracts to detect endogenous interaction: indeed, in β -catenin immunoprecipitates from differentiated Caco-2/TC7, the Mucdhl antibody detected a band of 130 kDa corresponding to the size of one of the Mucdhl M bands (Figure 7B). The presence of β -catenin and Mucdhl M in the same protein complex was further confirmed using in situ PLA,²⁶ a technique that detects protein interactions within intact cells by immunofluorescence: as shown on Figure 7C, coexpression of Mucdhl M and β -catenin in HEK293 cells generated a specific PLA signal that was slightly enhanced at the membrane (same results with HCT116, SW480 cells: not shown).

These results prompted us to examine whether Mucdhl M expression had any impact on β -catenin subcellular localization, which is critical for its activity. In cells ex-

pressing transiently Mucdhl M, we failed to detect a consistent change in the localization of β -catenin by immunostaining or subcellular fractionation (not shown). However, in HCT116 stable cells expressing Mucdhl M and grown in soft agar, the membrane localization of β -catenin appeared more pronounced (Figure 7D).

Finally, we examined whether this physical interaction interfered on the transcriptional activity of β -catenin. We performed luciferase assays, using the pTOPFLASH reporter that reflects nuclear activity of β -catenin.²⁷ In SW480 cells, which exhibit high constitutive activity of β -catenin because of the loss of APC function, expression of Mucdhl M, but not Mucdhl L, reduced the activity of pTOPFLASH and had no effect on the control reporter pFOPFLASH (Figure 7E and Supplementary Figure 5). In H293 cells, activity of pTOPFLASH induced by TCF4 and a stabilized β -catenin was clearly diminished by Mucdhl M and weakly by Mucdhl L (Figure 7F). Altogether, these data show that the interaction of Mucdhl M with β -catenin results in decreased β -catenin transcriptional activity.

Discussion

Mucdhl exhibits a restricted pattern of expression, with the intestinal epithelium being a major spot of synthesis.²¹ Given that members of the cadherin superfamily mediate critical interactions for organ morphogenesis and homeostasis, we explored the mechanisms regulating the expression of Mucdhl and the function of its 2 main isoforms in intestinal cells. The results obtained indicate that Mucdhl is associated with some of the key players of intestinal biology and tumorigenesis, ie, Cdx2 and β -catenin.

The starting point of this study was that Mucdhl was differentially expressed in samples from 2 types of experimental models having in common the modification of Cdx2 expression. A limited number of genes had their expression modified in both models, probably because they involved different cell lines and changes of numerous genes besides Cdx2. Most down-regulated genes were associated with cell cycle or DNA replication, whereas up-regulated genes were associated with the intestinal phenotype (adhesion molecules, enzymes). Given that LI-cadherin, a previously identified Cdx2 target,²⁸ was among these common genes, we hypothesized that Mucdhl might be a target of Cdx2. Indeed, we gathered compelling evidence that Cdx2 directly controls the transcription of Mucdhl in intestinal cell lines and identified the Cdx2-binding sites on the Mucdhl promoter. Importantly, the expression pattern of Mucdhl in the intestine was compatible with a regulation by Cdx2. Indeed, Mucdhl is predominantly expressed in differentiated cells of the intestine, and this expression is drastically reduced in physiopathologic conditions involving decreased Cdx2 expression (foregut-type heterotopia of Cdx2^{+/-} mice and colon tumors). Of note, microarray-based analysis of ileum samples from E18.5 embryos in which Cdx2 was homozy-

gously inactivated in the endoderm also predicted a strong decrease of Mucdhl expression.⁹ Altogether, these results strongly support the involvement of Cdx2 in the intestinal expression of Mucdhl. However, it is very likely that other transcription factors are also required because Mucdhl and Cdx2 expressions do not perfectly match in the gut (Cdx2 exhibiting earlier and wider expression) and because the level of stimulation of Mucdhl expression by Cdx2 depends on the cell line considered (Figure 1E). Nevertheless, Cdx2 is the first transcriptional regulator of Mucdhl identified to date.

We started to investigate the function and mode of action of Mucdhl in intestinal cells. In contrast to Cdx2,¹⁴ ectopic expression of Mucdhl L or M had no effect on cell growth in standard conditions of culture. However, in colony formation assays, expression of Mucdhl M drastically reduced the formation and maintenance of the colonies. In addition, colony formation in soft agar, which involves anchorage-independent and tridimensional growth in addition to single-cell growth, was almost abolished upon Mucdhl M expression and clearly reduced upon Mucdhl L expression. The mechanism of colony inhibition remains unclear. A direct effect on proliferation induced by the low amount of cells (ie, lack of secreted growth factor) is possible. Mucdhl may also impact on cell survival in stringent conditions because even established colonies collapsed upon Mucdhl M expression. Of note, cleaved Caspase 3 (indicative of apoptosis) was not detected in these conditions. Another possibility is that alteration of intercellular adhesion potentially induced by overexpression of Mucdhl leads to cell death as reported for other molecules.²⁹ This hypothesis is supported by (1) the observation that Mucdhl M-expressing colonies appeared irregularly shaped and were formed of oddly organized cells and (2) the fact that expression of Mucdhl L in fibroblasts induced their aggregation.¹⁹ However, in our cells, Mucdhl had no effect in aggregation assays (not shown). Finally, the unusual predominant apical location of Mucdhl in vivo prompted Goldberg et al¹⁹ to suggest that glycosylated Mucdhl polarizes the cell, and thus overexpression may disorient the cells within the colony. The observation that the impact of Mucdhl was more striking in soft agar assays may reflect additional loss of polarity clues because of the absence of anchorage and/or increased cellular stress. Future studies will clarify the process induced by Mucdhl, but it is noted that the possible mechanisms are matching several of Cdx2's cellular activities.^{10,14-18}

Concerning the mode of action of Mucdhl, we clearly showed that Mucdhl M can directly interact with β -catenin as suggested by colocalization of the 2 molecules at the membrane (Supplementary Figure 6). This colocalization was first detected by others in kidney cells,¹⁹ then more clearly in intestinal cells,³⁰ and finally confirmed in the gut.³¹ In addition, we found that overexpression of Mucdhl M reduced the transcriptional activity of β -catenin. The most obvious scenario is that the binding of Mucdhl M prevents the nuclear translocation of

β -catenin, although we only observed such trapping in Mucdhl M-expressing colonies grown in soft agar. However, recent data indicated a correlation between Mucdhl expression and membrane retention of β -catenin in colon tumors.³¹ Other possibilities that should be investigated in the future include modification of β -catenin phosphorylation, stability, or interaction with partners. Meanwhile, several important aspects can already be taken into consideration: (1) both the expression and activity of Cdx2 have previously been connected to β -catenin,^{32–34} indicating a strong interdependence between these important actors of the intestinal physiopathology; (2) Mucdhl is predominantly expressed in differentiated cells of the intestine, revealing an inverse correlation with proliferation and transcriptional activity of β -catenin³⁵; (3) the interaction with β -catenin essentially concerned Mucdhl M, indicating for the first time that both isoforms are not functionally equivalent. This aspect is in line with the lower efficiency of Mucdhl L in reducing colony formation but was unexpected because β -catenin interacts with the intracellular domain of cadherin molecules. The presence of the Mucin domain may modify the conformation of Mucdhl L or the binding to other molecules, thus preventing interaction with β -catenin. In any case, it is very likely that the activity of Mucdhl isoforms is also mediated by β -catenin-independent mechanisms, as indicated by the inhibitory effect of Mucdhl L on colony formation in soft agar and tumor formation in vivo. The potential SRC Homology 3 domain- or PSD-95/Discs Large/Zona Occludens 1 domain-binding motifs in the intracellular part of Mucdhl may participate to its signaling activity.

Finally, we found that Mucdhl expression was reduced in experimentally induced murine colon tumors as well as in most samples of a small collection of human colon cancers. Similar results were obtained in parallel with a bigger cohort of patients.³¹ This loss of Mucdhl might have functional consequences given that forced expression of Mucdhl opposed tumor formation in nude mice. Interestingly, decreased expression of Cdx2 in invasive colon cancer cells correlates with increased nuclear localization of β -catenin,³⁶ suggesting that the transition from a differentiated phenotype (polarity, adhesion) toward a more mesenchymal or stem cell-like phenotype contributes to the plasticity required for metastasis.³⁷ Altogether, these important observations warrant further investigation of Mucdhl in the gut.

Supplementary Materials

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.12.037.

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Conflicts of interest

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Supplementary Materials and Methods

Cell Lines

Caco-2/TC7 cells¹ were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 20% heat-inactivated fetal bovine serum (Invitrogen), 1% essential amino acids, and antibiotics. Human colon adenocarcinoma HCT116, HT29, T84, and SW480 cells (American Type Culture Collection) and embryonic kidney HEK293 (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics.

Plasmids

pRL-null was from Promega (Madison, WI) and pEGFP-C1 from Clontech Laboratories, Inc (Mountain View, CA). pcDNA6/TR and pcDNA4/TO were from Invitrogen (Carlsbad, CA). pTOPFLASH and pFOPFLASH,² pcDNA1 expressing Myc-tagged TCF4, and pCI-Neo expressing human β -catenin (wild-type [WT] or S33Y) were from H. Clevers (Utrecht University Medical Center, Utrecht, The Netherlands). pcDNA3 plasmids expressing Flag-tagged human β -catenin (WT or S33Y) were from Addgene (Cambridge, MA; No. 16828 and No. 19286, Kolligs et al³).

pcDNA4/TO-DHLM/Flag and pcDNA4/TO-DHLL/HA allow inducible expression of C-terminally Flag- or HA-tagged human Mucdhl M (NP_112554.2) and Mucdhl L (NP_068743.2), respectively. To generate pcDNA4/TO-DHLM/Flag, a *Sall*-*Bgl*II-digested polymerase chain reaction (PCR) fragment of 2026 base pair (bp) was first obtained using the PCRx Enhancer System (Invitrogen), a Mucdhl M-containing plasmid (clone IRATp970E11112D; Source BioScience LifeSciences, Nottingham, United Kingdom) as template, and Mucdhl M specific primers including the sequence for the Flag tag (TTG CTG AGA TCT CTG CCA GGT GAC AGC CGC CAA GATG and GCT AGC GCG TCG ACT TAC TTG TCG TCT TTG TAG TCG ATG TAG GAG TCA TCA CCA CCG G) and then subcloned into *Bam*HI/*Xba*I of pcDNA4/TO. To generate pcDNA4/TO-DHLL/HA, a fragment containing part of human Mucdhl L coding sequence (post M/L splicing site) obtained by reverse-transcription RT-PCR using Caco2/TC7 RNA and Mucdhl L specific primers including the sequence for the HA tag (CTG ACC ACC ACC ACA CTG GCA CAG GCG and TTA TCT AGA TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA GAT GTA GGA GTC ATC ACC ACC GGG CGC) was first subcloned into pCRII/TOPO (Invitrogen); a 1310-bp *Bst*XI-*Xba*I fragment from the resulting construct was switched with the 731-bp *Bst*XI-*Xba*I fragment of pcDNA4/TO-DHLM/Flag to generate pcDNA4/TO-DHLL/HA. To obtain pcDNA4/TO-DHLM/HA, the 587-bp *Xba*I-*Afe*I fragment of pcDNA4/TO-DHLL/HA containing the HA tag sequence was switched with the corresponding fragment in pcDNA4/TO-DHLM/Flag.

pFLAG-hCdx2 was obtained by first subcloning a reverse-transcription PCR fragment containing Cdx2 into pCRII/TOPO (Invitrogen) and next inserting a *Hind*III-*Xba*I fragment from the resulting plasmid into pFLAG-CMV2 (Sigma, St. Louis, MO).

To obtain WT pMucdhl-Luc constructs, 2.8- and 1.4-kilobase (kb) DNA fragments generated by PCR using T84 cells genomic DNA, the PCRx Enhancer System (Invitrogen), and Mucdhl specific primers including the sequence for *Nhe*I and *Kpn*I restriction sites (2.8 kb forward: TAT CGG TAC CCC TGA CCT TCC CGG GAT CGC T; 1.4 kb forward: TAC AAG GTA CCA AGG AGG AAT CAG ACC CAG ACG; reverse for both: TAC TAG CTA GCT TGG CGG CTG TCA CCT GGC AG) were first subcloned into pCRII/TOPO (Invitrogen) and next transferred into pGL3-basic (Promega) using *Kpn*I and *Nhe*I. Mutated pMucdhl-Luc plasmids were generated with the Quikchange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using first pMucdhl (1.4 kb)-Luc as template: m1-pMucdhl (1.4 kb)-Luc exhibited a mutation of the most proximal Cdx2 putative binding site (−105: ATAAA switched to GTGCA using GAA GCC GGT CGT AAA GGA TCG TGC ACT GGG CGG CGT CTG and CAG ACG CCG CCC AGT GCA CGA TCC TTT ACG ACC GGC TTC primers) and m2-pMucdhl (1.4 kb)-Luc exhibited a mutation of the less proximal Cdx2 putative binding site (−114, TAAA switched to AGTA using CTT GGC AGA AGC CGG TCG AGT AGG ATC ATA AAC TGG GCG and GAA CCG TCT TCG GCC AAC TCG TCC TAG TAT TTG ACC CGC primers) m1m2-pMucdhl (1.4 kb)-Luc, which is mutated on both Cdx2 consensus binding sites, was generated by using m1-pMucdhl (1.4 kb)-Luc as template and the m1m2 mutagenesis primer pair (TGG CAG AAG CCG GTC GAG TAG GAT CGT GCA CTG GG and CCC AGT GCA CGA TCC TAC TCG ACC GGC TTC TGC CA). Each new construct was checked by sequencing (GATC Biotech, Konstanz, Germany).

RT-qPCR and Microarray Analysis

For RT-qPCR, RT was performed in 20 μ L using the High Capacity Complementary DNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and 2 μ g of RNA. Diluted RT products (5–10 \times) were mixed with TaqMan Master Mix (Applied Biosystems) and gene-specific TaqMan probe and primers sets (TaqMan Gene Expression assays; Applied Biosystems) to perform quantitative PCR using the 7500 real-time PCR System (Applied Biosystems). The following TaqMan Gene Expression assays were used: Mucdhl, Hs01059246_m1 (detecting both isoforms); Cdx2, Hs00230919_m1; TBP, Hs99999910_m1; SI, Hs00356112_m1). Analysis of the results (duplicates) was performed with the 7500 software v2.0.1 (Applied Biosystems) using the relative $\Delta\Delta$ Ct quantification method.

Exploratory microarray-based experiments were performed in triplicates at the Microarray and Sequencing

Platform of the IGBMC (Strasbourg, France) using spotted high-density human 25 K oligonucleotide arrays.⁴

Chromatin Immunoprecipitation

Fixation of HCT116 cells was performed with 1% (vol/vol) formaldehyde for 10 minutes at room temperature and quenched with 0.125 mol/L glycine for 5 minutes. Chromatin immunoprecipitation experiments were carried out using the EZ-Magna ChIP G Chromatin Immunoprecipitation kit as recommended by the supplier (Millipore, Billerica, MA). Sheared cross-linked chromatin from $\sim 10^6$ cells was incubated overnight at 4°C with 1 μ g of mouse immunoglobulin G (IgG) (Upstate Cell Signaling) or with mouse anti-Cdx2 antibody (Biogenex, San Ramon, CA). Input corresponds to nonimmunoprecipitated sheared cross-linked chromatin from $\sim 10^5$ cells (1%). PCR analysis was performed with Taq PCRx DNA Polymerase (Invitrogen), 1/25th of immunoprecipitated DNA as template, and primers located in the human Mucdhl promoter (forward: ACC CAT GAG CCG CTC TCC AGT C; reverse: TCT GGC GTC TAG GAC TGG CGC AGT; annealing: 55°C; product: 248 bp) or the SI promoter (forward: GGC TGG TAA GGG TGC AAT AA; reverse: GCC TGT TCT CTT TGC TAT GTT G; annealing: 55°C; product: 119 bp).

Immunoprecipitation and Western Blot

Cells were treated or not with MG132 before lysis in 2% NP40-150 mmol/L NaCl-50 mmol/L-Tris pH 7.5 lysis buffer containing protease inhibitors (Complete; Roche Applied Science, Germany). For exogenous immunoprecipitation, 1 mg of cellular extract was incubated 2 hours at 4°C with 30 μ L of anti-HA affinity matrix (Roche). For endogenous immunoprecipitation, 1 mg of cellular extract was incubated overnight at 4°C with 1 μ g of antibody β -catenin (clone 14; BD Biosciences, Mountain View, CA) or mouse Ig (Millipore), and 30 μ L of protein G-Agarose beads (Roche) was added the last 45 minutes. In both cases, beads were next washed 4 times for 10 minutes with lysis buffer adjusted to 500 mmol/L NaCl, resuspended in 20 μ L of Laemmli loading buffer (Euromedex, Strasbourg, France), and boiled for 5 minutes before SDS-PAGE.

Western blot analysis was performed as described⁵ with one of the following primary antibodies: Cdx2 (1/5000; Biogenex), β -catenin (clone 14, 1/5000; BD Biosciences), Flag (clone M2, 1/2000; Sigma), Gapdh (1/3000),⁶ Mucdhl (H-225, 1/3000; Santa Cruz Biotechnology, Santa Cruz, CA), Mucdhl (C-20, 1/500; Santa Cruz Biotechnology), and Actin (1/15,000; Millipore).

Immunohistochemistry and Immunofluorescence

H&E staining and immunohistochemistry were performed as described⁷ using anti-Cdx2 (1/500; Biogenex), anti-Mucdhl (H-225, 1/500; Santa Cruz Biotech-

nology), or anti- β -catenin (clone 14, 1/200; BD Biosciences) antibody. Murine tissues were from WT, APC ^{Δ 14/+},⁸ or Cdx2^{+/-},⁹ mice treated or not with azoxymethane as described.¹⁰ Human samples were from the collection of the University Hospital of Strasbourg (Strasbourg, France).

Immunofluorescence was performed on fixed (4% paraformaldehyde, 15 minutes), paraffin-embedded sections of soft agar-grown colonies. After antigen retrieval (10 minutes in 10 mmol/L sodium citrate, pH 6), slides were blocked (5% Normal Goat Serum-0.1% Triton-phosphate buffered saline, 1 hour at room temperature), incubated overnight at 4°C with diluted primary antibodies (1/250° Mucdhl H-225 from Santa Cruz Biotechnology and 1/125° β -catenin clone 14, from BD Biosciences), washed 3 times for 5 minutes with 0.1% Triton-phosphate-buffered saline, incubated 1 hour at room temperature with diluted secondary antibodies (1/1000° Alexa Fluor 488 goat anti-mouse IgG and 1/1000° Alexa Fluor 568 donkey anti-rabbit IgG), washed again, stained for 4'-6-diamidino-2-phenylindole (DAPI, Sigma), and mounted with FluorSave Reagent (Millipore). Imaging was based on optical sectioning using the ApoTome system (Zeiss) and the ImageJ software (National Institutes of Health, Bethesda, MD).

Deglycosylation

Cells were lysed in 2% NP40-150 mmol/L NaCl-50 mmol/L-Tris pH 7.5 lysis buffer containing protease inhibitors (Complete; Roche). Deglycosylations were performed at 37°C on 10 μ g of protein extract. To remove N-glycosylation, extracts were incubated 2 hours with PNGase F (native; NEB, Ipswich, MA) as recommended. To remove both N and O-glycosylation, extracts were in addition treated overnight with Neuraminidase and Endo-a-N-Acetylgalactosaminidase (both NEB) as recommended.

Proliferation in Standard Conditions

Growth of cell population in standard conditions (\pm doxycycline) was evaluated by performing MTS assays (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega) at different time points after seeding 10000 cells/well in 96-well plates on day 1 (6 replicates).

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ANNEX 2

ETAT DE LA QUESTION

Les cancers colorectaux représentent un problème majeur de santé publique en raison de leur gravité et de leur incidence en constante augmentation. La gravité est essentiellement due à la résistance à la chimiothérapie et à la propension à la dissémination métastatique. Les modifications d'expression transitoires détectées dans certaines cellules cancéreuses constituent un aspect de l'hétérogénéité tumorale et reflètent/conditionnent la plasticité nécessaire à la dissémination métastatique.¹ **Ces modifications pourraient représenter de nouvelles cibles thérapeutiques, mais sont à l'heure actuelle peu caractérisées, notamment en raison de l'absence de modèle expérimental approprié.**

Dans les tumeurs coliques, le gène suppresseur de tumeurs Cdx2 présente fréquemment une expression hétérogène. En particulier, l'expression de Cdx2 est absente des cellules invasives. Par contre, l'expression de Cdx2 est détectée dans les métastases, ce qui suggère l'existence d'un processus dynamique dépendant du micro-environnement tumoral.² Ce facteur de transcription homéotique détermine, entre autres, l'identité intestinale pendant l'embryogenèse³ et contribue à l'homéostasie de l'épithélium intestinal chez l'adulte⁴. Son expression dans les cellules cancéreuses coliques induit leur différenciation⁵, inhibe leur prolifération, leur migration in vitro et diminue leur dissémination métastatique in vivo.⁶ **Les relais moléculaires de l'activité anti-tumorale de Cdx2 ne sont encore que partiellement définis, mais ce gène, par son profil d'expression et sa fonction, permet d'appréhender les notions d'hétérogénéité et de plasticité tumorales.**

QUESTIONS POSEES

I : Comment est-ce que la plasticité des cellules invasives influence la progression tumorale dans les tumeurs coliques ?

II: Quels sont les relais moléculaires de la fonction de Cdx2 ?

TRAVAUX REALISES

Question I - Plasticité et Progression Tumorale : Etablissement et Caractérisation d'un Modèle d'hétérogénéité Tumorale

Etant donné que Cdx2 peut être considéré comme un marqueur d'hétérogénéité intra-tumorale, mon objectif était de créer des cellules cancéreuses coliques rapportrices du niveau d'expression de Cdx2 en introduisant par 'knock-in' somatique la séquence codante de la GFP dans le locus Cdx2. Dans ces lignées la GFP reflètera le niveau d'expression de Cdx2, puisqu'elle sera traduite en phase avec la séquence codante de ce gène. Ces cellules 'rapportrices' seront injectées à des souris nude afin d'obtenir des tumeurs à partir desquelles les cellules seront triées sur la base du taux d'expression de Cdx2 mesuré par la fluorescence émise. Les différentes populations cellulaires seront caractérisées du point de vue de leur agressivité tumorale (greffes souris nude) et du point de vue moléculaire (transcriptomes par micropuces). En outre, ces cellules rapportrices pourraient être utilisées pour cribler une chimiothèque à la recherche de molécules capables de stimuler l'expression de Cdx2 dans les cellules cancéreuses coliques humaines.

➤ Approche expérimentale et résultats

Par transfection de cellules cancéreuses coliques humaines (T84 et HT29) et sélection au G-418, j'ai établi et validé deux lignées stables distinctes exprimant constitutivement la protéine fluorescente rouge Cherry (T84-Cherry et HT29-Cherry) : cette fluorescence permettra de collecter spécifiquement les cellules humaines greffées chez la souris. En parallèle, j'ai construit un vecteur de recombinaison homologue de type viral pour insérer la GFP dans le locus de Cdx2. Les HT29-Cherry ont été infectées par les particules virales et des clones résistants à l'antibiotique de sélection ont été isolés et amplifiés. J'ai ensuite examiné l'ADN génomique de 675 clones par PCR : 1 seul était positif pour l'insertion de la construction dans le locus de Cdx2 en 5' et 3'.

➤ Conclusions et perspectives

Pendant ma thèse j'ai pu réaliser la première étape de ce projet (construction du vecteur de recombinaison, lignées cellulaires). Avant d'utiliser la lignée établie, qui a intégré la construction knock-in dans le locus Cdx2, dans des expériences de greffes, il faudra valider ce clone in vitro (détection de la protéine de fusion Cdx2-GFP) et in vivo (plasticité de l'expression de Cdx2).

Question II - Détermination des Relais Moléculaires de Cdx2

A. Du gène cible à la fonction : Mucdhl, un nouvel acteur de l'homéostasie intestinale ?

Afin de comprendre comment Cdx2 exerce sa fonction anti-oncogénique, des analyses de transcriptome ont été réalisées au laboratoire afin d'identifier de nouveaux gènes cibles potentiels. Les résultats de ces analyses suggéraient que le gène Mucdhl était une cible de Cdx2. Mucdhl est une protéine atypique de la famille des molécules d'adhésion de type cadhérine et deux isoformes générées par épissage alternatif ont été détectées dans l'intestin. *Mon but était de déterminer si Cdx2 régule l'expression de Mucdhl et d'étudier la fonction de Mucdhl, inconnue à ce jour. Les résultats obtenus ont fait l'objet de la Publication #1.*

➤ **Approche expérimentale et résultats**

✓ Par des expériences de gain/perte de fonction de Cdx2, j'ai montré que la quantité de transcrits Mucdhl varie en fonction de l'expression de Cdx2. Il s'agit d'une régulation directe car j'ai montré par CHIP que Cdx2 se fixe sur les régions promotrices de Mucdhl. De plus, j'ai identifié les sites de liaison de Cdx2 en construisant et testant des vecteurs rapporteurs comprenant différents fragments des régions promotrices de Mucdhl en amont du gène de la Luciférase.

✓ Par immunohistochimie, j'ai déterminé le profil d'expression de Mucdhl dans l'intestin et constaté qu'il était compatible avec une régulation par Cdx2 in vivo. En outre, j'ai comparé l'expression de Mucdhl chez des individus sains et chez des individus atteints de cancers coliques: l'expression de Mucdhl semble fréquemment réduite dans les tumeurs coliques, ce que j'ai confirmé par RT-qPCR.

✓ Pour étudier la fonction de Mucdhl dans les cellules cancéreuses coliques, j'ai établi des lignées cellulaires stables qui expriment de manière inductible l'une ou l'autre des isoformes de Mucdhl. Par des tests in vitro, j'ai pu montrer que l'isoforme courte inhibe fortement la formation de colonies dans l'agarose mou et sur du plastique. Afin d'évaluer l'impact de Mucdhl sur les cellules cancéreuses in vivo, j'ai injecté les lignées stables sous la peau de souris nude et observé que les deux isoformes de Mucdhl inhibent la croissance tumorale.

✓ A la recherche d'un relais moléculaire pour cet effet, j'ai montré par co-immunoprécipitation et PLA in situ que l'isoforme courte de Mucdhl interagit avec la β -caténine et inhibe également son activité transcriptionnelle dans des tests luciférase basés sur le vecteur rapporteur Topflash.

➤ **Conclusions et perspectives**

Ce travail représente la première étude sur la régulation de Mucdhl et la plus complète sur sa fonction. J'ai pu montrer que cette cadhérine a un effet anti prolifératif sur les cellules cancéreuses coliques in vitro et in vivo. Ainsi Mucdhl pourrait être un des effecteurs de la fonction de suppresseur

de tumeurs de Cdx2. En outre, il interagit avec la β -caténine qui joue un rôle crucial dans la dissémination métastatique. Par la suite, il faudra d'une part déterminer la fonction et le mode d'action de Mucdhl dans l'épithélium intestinal et d'autre part vérifier qu'il agit comme suppresseur de tumeurs coliques.

B. De la fonction aux gènes cibles: le renforcement de la rigidité tissulaire via les Rho-GEFs, un obstacle à la dissémination métastatique ?

En parallèle, l'approche inverse, basée sur propriétés morphologiques et fonctionnelles induites par Cdx2 dans les cellules cancéreuses coliques, a été entreprise. *Le but de ce travail était de déterminer si Cdx2 impacte la rigidité des cellules cancéreuses, qui pourrait s'opposer à la dissémination métastatique, et de décortiquer les mécanismes moléculaires impliqués dans ce processus. Les résultats obtenus feront l'objet de la Publication #2.*

➤ **Approche expérimentale et résultats**

✓ Pour étudier les effets de Cdx2 sur les cellules cancéreuses coliques, une ligné cellulaire stable avec expression inductible de Cdx2 a été créée. Dans le cadre d'une collaboration, il a été montré par microscopie à force atomique que les cellules qui expriment Cdx2 forment un tapis nettement plus rigide que les cellules contrôles. Par immunofluorescence, nous avons observé que cette rigidité accrue corrèle avec un renforcement des jonctions adhérentes et une réorganisation spectaculaire du cytosquelette d'actine en fibres de stress robustes et bien alignées. L'utilisation d'inhibiteurs et de tests d'activité, nous a permis de mettre en évidence l'activation des cascades de signalisation Rho/ROCK/MLC et Rho/Dia1 en présence de Cdx2.

✓ Afin de trouver les gènes cibles de Cdx2 à l'origine de ces processus, j'ai examiné par RT-qPCR l'expression d'une sélection de gènes impliqués dans l'organisation du cytosquelette en présence ou en absence de Cdx2. De la même manière, j'ai analysé l'expression des 28 Rho-GEFs (activateurs de Rho) identifiés à ce jour. Cette stratégie m'a permis d'identifier 2 gènes cibles potentiels qui codent pour des Rho-GEFs : MCF2/dbl et Vav3.

✓ J'ai montré par des expériences de gain/perte de fonction de Cdx2 que les transcrits des deux isoformes de Vav3 varient en fonction de l'expression de Cdx2. En outre, j'ai réalisé des expériences de CHIP et détecté la fixation de Cdx2 sur les régions promotrices des 2 isoformes de Vav3. Enfin, dans des tests Luciférase, Cdx2 stimule l'activité de ces régions promotrices.

➤ **Conclusions et perspectives**

J'ai montré qu'un régulateur du cytosquelette, Vav3, est régulé par Cdx2. Par la suite, il s'agira de démontrer formellement que Vav3 est l'effecteur de Cdx2 pour l'activation de RhoA et la formation

des fibres de stress par des études fonctionnelles et de déterminer le rôle de Vav3 dans l'épithélium intestinal sain et pathologique.

CONCLUSION GENERALE

Mon travail de thèse a permis de mieux appréhender le mode d'action de Cdx2 en tant que suppresseur de tumeurs. L'identification et la caractérisation de deux nouveaux gènes cibles de Cdx2 mettent en lumière son implication dans le phénotype épithélial, notamment au niveau de la cohésion tissulaire (contacts cellulaires et rigidité). Après validation, le modèle d'hétérogénéité tumorale (lignée rapportrice de l'expression de Cdx2) que j'ai construit pourrait constituer un outil précieux pour caractériser à plus grande échelle les événements moléculaires et cellulaires associés à l'hétérogénéité tumorale. Au-delà des cibles de Cdx2, ceci devrait ouvrir la voie à : i) la validation de marqueurs moléculaires diagnostiques/pronostiques des tumeurs coliques ; ii) l'identification de gènes ou de voies de régulation susceptibles de constituer de nouvelles cibles thérapeutiques de drogues visant à limiter la plasticité tumorale. En complément de la résection chirurgicale et de la chimiothérapie avec des agents cytotoxiques, ces drogues devraient améliorer l'espérance de vie des patients.

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Plasticité des Cellules Cancéreuses Coliques:

Impact du Facteur d'Identité Tissulaire Cdx2

Résumé

Le facteur de transcription homéotique Cdx2 est un suppresseur de tumeurs et son expression est réduite de manière hétérogène dans les tumeurs coliques. Or, le cancer colorectal demeure un problème de santé publique par sa fréquence et sa gravité.

Au travers de 3 sous-projets, cette thèse visait à comprendre le mode d'action de Cdx2. Premièrement, la cadhérine Mucdhl a été identifiée et caractérisée en tant que nouvelle cible de Cdx2 : Mucdhl inhibe la croissance des cellules cancéreuses coliques et s'oppose à l'activité de la β -caténine. Deuxièmement, un effet inattendu de Cdx2 sur le cytosquelette et la rigidité cellulaire a été montré. Ceci pourrait expliquer comment Cdx2 intervient dans l'organisation structurale des entérocytes ou la migration. En parallèle, une lignée cellulaire rapportrice de l'expression de Cdx2 a été créée qui, après validation, sera un outil précieux pour l'étude des mécanismes moléculaires qui conditionnent l'hétérogénéité tumorale.

Par la mise en évidence de nouvelles fonctions et cibles de Cdx2, cette thèse permet de mieux appréhender son rôle physiologique et son action de suppresseur de tumeurs dans l'intestin.

Mots clés : Cdx2, Cancer colorectal, Mucdhl, Intestin, Cadhérine, Cytosquelette, Recombinaison homologue somatique, Vav3

Résumé en anglais

Colorectal cancer is a major public health problem because of its frequency and propensity to metastasize. Cdx2 plays the role of a tumor suppressor and its expression is frequently but heterogeneously reduced in colon tumors.

Through three sub-projects, this thesis aimed to better understand the mode of action of Cdx2. First, the Cadherin Mucdhl was identified and characterized as a new direct target gene of Cdx2. Mucdhl was also shown to inhibit the growth of colon cancer cells and to oppose β -catenin activity. Second, an unexpected effect of Cdx2 on the cell cytoskeleton and rigidity of a cancer cell monolayer was uncovered. This gives new clues about how Cdx2 contributes to the structural organization and differentiation of enterocytes but also inhibits cell migration. In parallel, a reporter cell line for Cdx2 expression was created. After its validation, this cell line will be a precious tool to study the molecular mechanisms underlying tumor heterogeneity in vivo.

Altogether, this thesis unraveled new functions and target genes of Cdx2 that permit to better apprehend its physiological function and its action as a tumor suppressor in the intestine.

Key words: Cdx2, Colorectal cancer, Mucdhl, Intestine, Cadherin, Cytoskeleton, Somatic homologous recombination, Vav3