Faculty of Medicine of the University of Porto
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Regulation of CDX2 and intestinal differentiation in homeostasis and carcinogenesis: emphasis on the role of MEX3A

Regulação do CDX2 e diferenciação intestinal em homeostasia e carcinogénese: relevância funcional do MEX3A

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ABSTRACT

The homeodomain transcription factor CDX2 is a key player in intestinal differentiation. Therefore, it is not surprising to find its expression significantly altered in carcinogenic processes of the gastrointestinal tract. In the stomach, *de novo CDX2* expression drives a preneoplastic lesion known as intestinal metaplasia that increases the risk of gastric cancer development. Quite the opposite, in the context of colorectal cancer, CDX2 has been classically described as a tumour-suppressor, although this view has been gradually challenged in the last few years. Notwithstanding, it is known that tumours retaining CDX2 expression are more differentiated and show better outcome in terms of survival. Thus, a comprehensive understanding of CDX2 regulation stands out as a research priority, for in the long-run, the acquired knowledge could be integrated into alternative therapeutic strategies that might benefit the patient. Taking into consideration that the extracellular microenvironment is also important to the cellular malignant behavior as genetic constrains, we decided to tackle its influence over CDX2 expression in two fronts:, analyze the possible role of DNA epigenetic changes, hypothesizing that infection by *Helicobacter pylori* and the generation of chronic inflammation could in some way affect the *CDX2* gene methylation pattern and induce its transcription; and in a more exploratory approach, establish 3D culture models that better mimic the *in vivo* milieu, hypothesizing that they would provide new insights regarding signaling pathways affecting CDX2 levels. We show here that there is a lack of correlation between *CDX2* methylation status and its expression in intestinal metaplasia and gastric cancer cell lines, excluding this mechanism as a *CDX2* regulatory feature during gastric malignancy. On the other hand, through genome-wide screening of the 3D culture system, comprising a gastric cancer cell line with CDX2 expression and the basement matrix matrigel, we were able to identify the RNA-binding protein MEX3A as putatively involved in CDX2 regulation. Further supported by an evolutionary functional link to its *Caenorhabditis elegans* orthologue MEX-3, we demonstrate that MEX3A maintains a conserved repressive function over CDX2 in gastric and intestinal cellular models. This is dependent on the interaction with a specific binding determinant present in *CDX2* mRNA 3`untranslated region. Moreover, MEX3A overexpression carries phenotypic consequences, impairing intestinal differentiation and cellular polarization, while promoting gain of stemness properties. As a result, we describe for the first time a

mechanism of CDX2 post-transcriptional control, likely contributing to intestinal homeostasis and carcinogenesis, and add a new layer to the already intricate CDX2 regulatory network.

RESUMO

O factor de transcrição homeobox CDX2 é um elemento crítico na diferenciação intestinal. Assim, não é inesperado o facto de apresentar níveis de expressão significativamente alterados em processos carcinogénicos do tracto gastrointestinal. No estômago, a expressão *de novo* de CDX2 induz uma lesão pré-neoplásica denominada metaplasia intestinal que confere um risco acrescido de desenvolvimento de carcinoma gástrico. Pelo contrário, no contexto colorectal, este gene tem sido geralmente descrito como um supressor tumoral, embora esta classificação tenha vindo a ser posta em causa nos últimos anos. Mesmo assim, reconhece-se que tumores com expressão de CDX2 são mais diferenciados e detêm melhor prognóstico em termos de sobrevida. Torna-se, então, fundamental obter uma compreensão aprofundada dos processos de regulação de CDX2, pois a longo prazo o conhecimento adquirido pode ser integrado em estratégias terapêuticas alternativas que poderão beneficiar o paciente. Tendo em consideração que o microambiente extracelular é, tal como as alterações genéticas, importante para o comportamento celular maligno, decidimos confrontar a sua influência sobre a expressão de CDX2 em duas partes: analisar o possível papel de alterações epigenéticas ao nível do promotor do gene, colocando a hipótese de que o processo de infecção por *Helicobacter pylori* e a subsequente resposta inflamatória poderão criar condições que afectam o perfil de metilação de *CDX2* induzindo a sua transcrição; e numa abordagem de carácter mais exploratório, estabelecer modelos de cultura 3D que mimetizem o ambiente *in vivo*, colocando a hipótese de que estes permitiriam obter novos dados no que respeita às vias de sinalização que afectam os níveis de CDX2. Demonstrámos que não existe uma correlação entre o estado de metilação do promotor de *CDX2* e a sua expressão na metaplasia e em linhas celulares de cancro gástrico, excluindo este mecanismo de regulação no processo de carcinogénese gástrica. Por outro lado, através de um varrimento transcricional do sistema de cultura 3D, constituído por uma linha celular gástrica e a matriz de membrana basal matrigel, identificámos uma proteína de ligação ao ARN designada MEX3A como estando potencialmente envolvida na regulação de CDX2. Apoiados numa base funcional evolutiva em relação ao papel do ortólogo MEX-3 em *Caenorhabditis elegans*, provámos que o MEX3A mantém uma função conservada enquanto repressor da expressão de CDX2 em modelos celulares gástricos e intestinais. Este efeito é dependente da

interacção com um motivo de ligação específico presente na região 3` não-traduzida do ARN mensageiro de *CDX2*. Adicionalmente, a sobrexpresão de MEX3A acarreta consequências fenotípicas, afectando a diferenciação intestinal e polaridade celular, promovendo simultaneamente o ganho de propriedades estaminais. Em conclusão, descrevemos pela primeira vez um mecanismo pós-transcricional de controlo de CDX2, com relevância para a homeostasia intestinal e provavelmente carcinogénese, adicionando um novo elemento à complexa rede de regulação do gene CDX2.

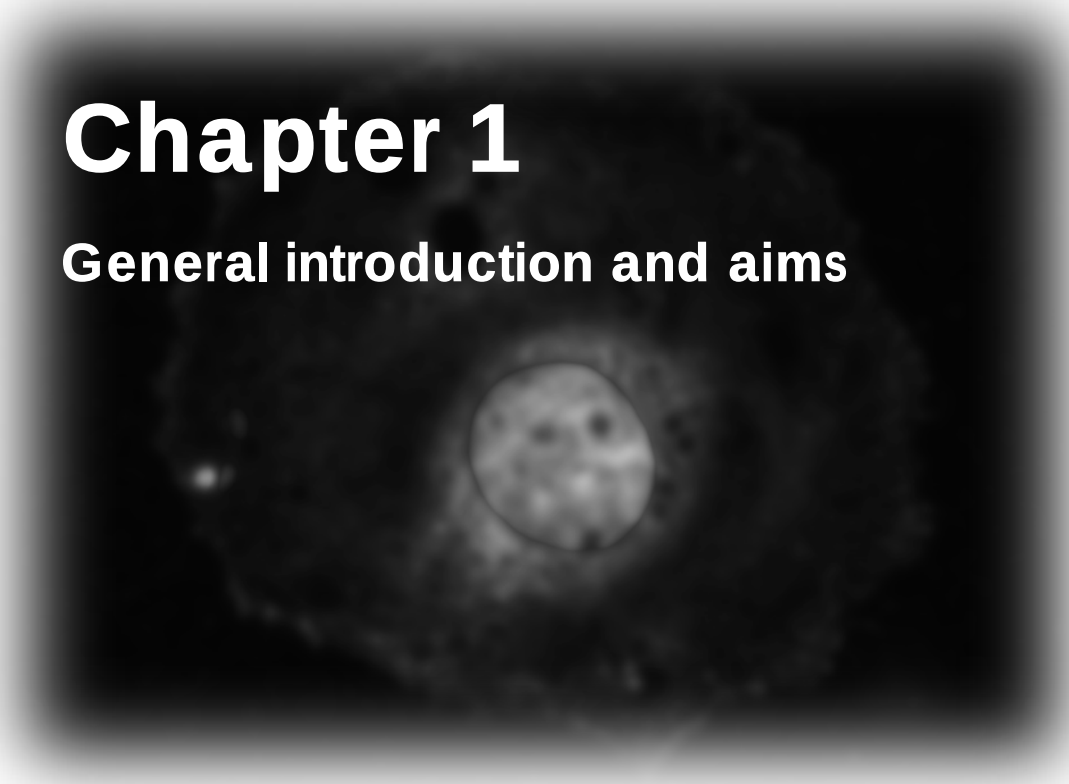
ABBREVIATIONS

The following list describes relevant abbreviations and acronyms used.

Bcd	Bicoid
BMP	Bone morphogenetic protein
Cad	Caudal
CDX	Caudal type homeobox
CIN	Chromosomal instability
CpG	Cytosine guanine dinucleotide
CRC	Colorectal cancer
eIF	Eukaryotic initiation factor
GI	Gastrointestinal
GLD-1	Defective in germline development
hnRNP K	Heterogeneous nuclear ribonucleoprotein K
HOX	Homeobox
IM	Intestinal metaplasia
KH	K-homology
LGR5	Leucine rich repeat-containing G-protein coupled receptor 5
LRC	Label-retaining cell
mex	Muscle excess
MEX3	Mex-3 homologue
miR	microRNA
MRE	MEX-3 recognition element
mRNP	Messenger ribonucleoprotein
MSI	Microsatellite instability
MSI1	Musashi-1
OLFM4	Olfactomedin-4
P body	Processing body
QKI	Quaking
RBP	RNA-binding protein
RING	Really interesting new gene
RKHD	RING finger and KH domain-containing
TE	Trophectoderm
UTR	Untranslated region

Chapter 1

General introduction and aims



1.1 GASTROINTESTINAL CARCINOGENESIS

Gastrointestinal (GI) cancers are a leading cause of morbidity worldwide. Given the high number of affected patients, a tremendous amount of effort has been spent in the search to prevent and treat these diseases over the past decades. This section intends to summarize specific aspects of GI carcinogenesis, as the gastric and intestinal settings constitute the biological frame upon which this work was developed.

1.1.1 An overview on gastric cancer

Gastric cancer is currently the fourth most common cancer worldwide, as 8% of newly diagnosed cases are stomach malignancies (Ferlay *et al.* 2010). Its frequency varies greatly across different geographic locations, being Eastern Asia, Eastern Europe, and South America the areas with highest incidence. In spite of a steady decline over the past decades, more than 700.000 people still perish each year, making it the second leading cause for cancer-related deaths. In Portugal, it places fifth and third in terms of incidence and mortality, respectively (IARC 2010).

About 90 to 95% of malignant tumours of the stomach are epithelial in origin and classified as adenocarcinomas. According to the Lauren system, two major histological types can be described with distinct clinicopathological features: diffuse and intestinal (Lauren 1965). Diffuse-type cancers consist of poorly-cohesive cells diffusely infiltrating the gastric wall, with little or no gland formation. They occur more often in low-risk areas, prevalently in young and female patients, being associated with worse prognosis (Bosman *et al.* 2010). Intestinal-type cancers show recognizable gland formation that is similar in microscopic appearance to the intestinal mucosa, growing in an expanding, rather than an infiltrative pattern. It is more common in countries with high incidence rates, mainly in male and older patients, but its frequency is decreasing (de Vries *et al.* 2007).

The marked incidence variation observed according to geographic location and socio-economic status, even within the same country, demonstrates that a complex interplay between genetic variation and environmental exposures constitutes the basis for gastric cancer susceptibility. Whereas about 10% present familial aggregation, which includes hereditary diffuse gastric cancer, an autosomal-dominant syndrome associated with E-cadherin (*CDH1*) gene germline mutations

(Guilford *et al.* 1998), most gastric cancers occur sporadically. A heterogeneous genetic and epigenetic milieu with incremented activity of oncogenes, such as *ERBB2* amplification, inactivation of tumour suppressor genes like *TP53*, deregulation of signalling pathways like TGF-β, and microsatellite instability (MSI), has been observed in the context of gastric malignancies (Zheng *et al.* 2004a). Furthermore, it is evident that DNA polymorphisms for inflammatory cytokine genes like *IL1B*, along with individual immune function contribute to the disease (El-Omar *et al.* 2000). Epidemiological data also suggests that dietary and behavioural elements are determinant in gastric cancer aetiology. The most relevant risk factors include high salt intake, sustained cigarette smoking, and heavy alcohol consumption, while the intake of fruits and vegetables is probably protective (Guggenheim and Shah 2013). Nonetheless, the most important advance in gastric adenocarcinoma epidemiology was its association with *Helicobacter pylori* infection, culminating in the classification of the gram-negative bacterium as a type I carcinogen by the International Agency for Research on Cancer (IARC 1994), and on the recognition of the carcinogenic propensity different strains harbour. For example, the presence of virulence factors encoded by the *cag* pathogenicity island genes, such as CagA, are characteristic of strains carrying higher risk (Yamaoka 2010). Pelayo Correa`s groundwork defined a series of histological stages in the canonical progression to gastric adenocarcinoma that still stands today (Correa 1992). According to the pathway, bacterial infection and the previously mentioned constrains trigger a prolonged precancerous process that evolves sequentially through increasingly more severe

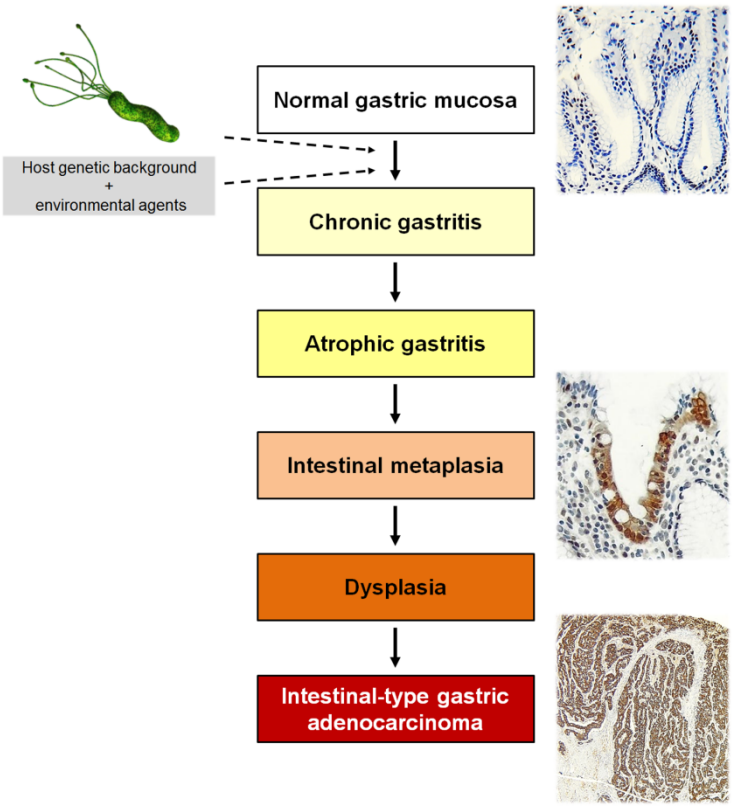


Figure 1. The gastric carcinogenic pathway. Representative immunohistochemistry images depicting global histology of normal gastric epithelium, an intestinal metaplastic gland and an intestinal-type gastric cancer invasive area.

lesions: chronic gastritis, multifocal atrophy, intestinal metaplasia (IM) and dysplasia. This multistage process transforms the normal gastric epithelium, ultimately leading to intestinal-type gastric cancer (**Figure 1**). Although *H. pylori* significantly increases the risk of developing both subtypes of gastric cancer, the diffuse-type typically progresses following chronic inflammation with no known precursor lesion, though silencing of *CDH1* gene is a key carcinogenic event.

H. pylori is estimated to infect close to half of the world`s population, making it the most widespread infection in the planet. Colonization of the human stomach can occur in early childhood, particularly in developing countries, and persists lifelong. In countries like Japan and Portugal, the prevalence of infection remains very high, reaching about 80% of the population. IM arises in approximately 30% of the infected individuals, from which only about 7% will progress to cancer (Uemura *et al.* 2001). Notwithstanding, the five-year survival rate for gastric cancer ranges from 10 to 30% (Dicken *et al.* 2005), since it is a largely asymptomatic disease at early stages. Therefore, it is often detected late, when already in advanced progression. Treatment modalities have been improving slowly and are currently limited, involving surgical resection, together with adjuvant and palliative chemotherapy-or radiotherapy-based regimens (Selgrad *et al.* 2010). The role of *H. pylori* eradication as the main preventive strategy continues to be controversial and largely dependent on the degree and extent of preneoplastic changes at the time of eradication (de Vries *et al.* 2007). New approaches for prevention and management are thus necessary, and early detection of gastric preneoplastic lesions stands in the front line as one of the most appealing strategies to ameliorate prognosis and increase survival.

1.1.2 Intestinal metaplasia

IM has been extensively studied as a putative preneoplastic lesion, and evidence of a clear association with stomach cancer development exists. An epidemiological study has demonstrated that IM is related with a six-fold increased comparative risk of malignancy and that 83% of gastric carcinomas arise in this setting (Uemura *et al.* 2001). This suggests a direct causal relationship, further supported by Mongolian gerbil rodent experimental models (Watanabe *et al.* 1998; Zheng *et al.* 2004b). The question of IM reversibility is crucial for clinical purposes, and

although conflicting, existing data points towards little evidence in its favour upon eradication of *H. pylori*, particularly under infection with high virulence strains (Barros *et al.* 2010), which makes the understanding of this lesion pathophysiology an even more imperative matter.

Metaplasias are general processes of cell- and tissue-type transformations (Slack 2007). These constitute fascinating phenomena, in the sense that they appear to disrupt our traditional conception of development as being hierarchically determined. Gastric IM is a prototype of glandular to glandular metaplasia and can be described as a multifocal cellular shift in the normal gastric gene expression background towards an intestinal-oriented genetic program, leading to the appearance of biochemically well-defined intestinal cell-types, alone or intermingled with gastric cells. As stomach and intestine arise from neighbouring territories of the endoderm during normal embryogenesis, IM presumably reflects this close developmental relationship. Nevertheless, it implies that certain embryological rulings must be circumvented or even erased upon specific microenvironmental stimuli (Tosh and Slack 2002).

There are conflicting theories regarding the starting point of this lesion. Some authors support the hypothesis of a transdifferentiation process, the switch of a differentiated gastric cell to a fully mature intestinal cell, eventually involving de(re)-differentiation steps (Kirchner *et al.* 2001; Goldenring *et al.* 2011). Assuming the transformed cells have inherent characteristics of an adult phenotype, with an already restricted lifespan, the biggest caveat in this theory is that these cell types may not survive long enough for the necessary epithelial self-renewal and maintenance demanded in the persistence of a continuous, though abnormal, regeneration process. So, most studies rely upon the hypothesis that IM is initiated by a new commitment pathway at the level of stem cells. The consensual view considers endogenous tissue-specific stem cells as being involved. The gastric epithelium is organized into numerous gastric units composed of flask-shaped tubular glands, several of which feed into a single pit that opens out onto the surface epithelium lined with mucous-secreting cells. Glands are constituted by various cell types located within three distinct regions denoted by the isthmus, neck, and base. Multipotent gastric stem or progenitor cells have been described to be located in the isthmus or base region, giving rise to all differentiated cells via complex migration patterns; consequently, gastric glands are monoclonal. Alternatively, other authors

propose that bone marrow-derived cells might be the initiator cells in the gastric carcinogenic process, including IM and dysplasia, by homing in and engrafting to the injury sites (Houghton *et al.* 2004; Varon *et al.* 2012). Although enticing as a theory, there has been limited translation of this model into the human setting. Whichever the case, IM is most probably the outcome of an adaptive response that goes amiss, in which the tissue tries to cope with several aggressive inputs, but ends up with the creation of susceptible ground for neoplastic transformation (Mesquita *et al.* 2006).

In terms of classification, two major gastric IM types can be recognized, taking into account not only morphological, but also molecular alterations, like loss and gain of mucinous differentiation markers and mucin-associated carbohydrate antigens (Reis *et al.* 1999; Silva *et al.* 2002). The complete IM or intestinal type (previously denominated type I) reflects a complete switch in the differentiation program, with loss of gastric mucins MUC1, MUC5AC, and MUC6, and *de novo* expression of the intestinal mucin MUC2. It is characterized by the presence of absorptive cells, Paneth cells and goblet cells secreting sialomucins, similar to the small intestinal phenotype. The incomplete IM or GI-mixed type reflects a mixture of gastric and intestinal components, both at the glandular and cellular level (Niwa *et al.* 2005). It is characterized by the presence of columnar and goblet cells secreting sialomucins and/or sulphomucins (previously denominated types II and III, respectively), similar to the colonic phenotype. Remarkably, the ectopic intestinal glands still preserve a normal cell migration pattern, which is achieved by a redeployment of the proliferative niche from the middle to the base of the gland (Inada *et al.* 2001; Sakamoto *et al.* 2011). Reciprocal interactions between the epithelium and the mesenchyme might be behind this architectural remodelling (Mutoh *et al.* 2005a), as the induction of a mesenchymal intestinal phenotype during the early stages of IM might lead to the establishment of a positive regulatory loop involving paracrine signals (Sakagami *et al.* 1984). It is not yet clear if the two IM types can be considered sequential steps in a shared process of gradual intestinalization or if they arise independently. The possibility exists that the incomplete type is biologically more unstable as it reflects an aberrant differentiation program without phenotypic parallel in the adult organism. In agreement, epidemiological data, though scarce, shows that it confers increased risk of gastric cancer development compared to the complete type (Filipe and Jass 1986; Rokkas

et al. 1991; Filipe *et al.* 1994), highlighting the importance of stratification concerning prognostic significance (González *et al.* 2013). Curiously, bacterial colonization is typically absent in foci of complete IM (Bravo and Correa 1999), favouring a presumably protective function.

1.1.3 Colorectal carcinogenesis

Colorectal cancer (CRC) is currently the third most common cancer worldwide, as 10% of the newly diagnosed cases are malignancies of the colon or rectum. Almost 60% of the cases occur in developed regions and the areas with highest incidence are North America, Australia/New Zealand and Europe (Ferlay *et al.* 2010). More than 600.000 people die each year, making it the fourth leading cause for cancer-related death. In Portugal, considering the total number of cases, it places first in terms of incidence and mortality (IARC 2010). As for the small intestine, it has a remarkably low incidence of primary carcinomas, especially considering its length and surface area, and those that do occur are often related to hereditary syndromes.

About 90% of sporadic tumours occur in individuals over the age of fifty. Other risk factors include family history, a diet low in fibers and high in red meat, alcohol, smoking, and sedentary occupation (Johnson *et al.* 2013). A present estimate is that 15-30% of CRCs have a familial component. Less than 5% of these happen in a recognizable setting of highly penetrant cancer syndromes due to germline mutations, being the most common the hereditary nonpolyposis CRC and familial adenomatous polyposis (Fearon 2011). Despite advances in surgical techniques and adjuvant therapy, there has been only a modest improvement in survival for patients with advanced neoplasms (Edwards *et al.* 2012). Hence, effective primary and secondary preventive approaches must be developed to reduce morbidity and mortality.

Since the description of the classic adenoma-carcinoma pathway (Fearon and Vogelstein 1990), defining CRC as the result of a gradual accumulation of changes that transform normal glandular epithelial cells into adenoma, followed by invasive carcinoma and eventually metastatic cancer, our understanding of its molecular pathogenesis has advanced and led to numerous revisions of this linear tumour progression model. It is now recognized that loss of genomic stability is a hallmark

feature of colorectal carcinogenesis (Grady and Carethers 2008), and this property led to the establishment of a classification system: (i) the chromosomal instability (CIN) phenotype, found in as many as 85% of tumours and defined by the presence of aneuploidy or structural aberrations; (ii) the MSI phenotype, defined by the presence of unstable loci due to inactivation of genes in the DNA mismatch repair family; and (iii) the CpG island methylator phenotype, exhibiting both global DNA hypomethylation and hypermethylation of gene promoters that contain CpG islands (Pritchard and Grady 2011). It is the accumulation of mutations combined with multiple cycles of clonal selection that results in the deregulation of signalling pathways controlling cell proliferation, differentiation, apoptosis, angiogenesis and invasion, ultimately culminating in cancer development.

1.1.4 Intestinal homeostasis

The intestinal tract is anatomically divided into two well-defined segments, the small intestine and large intestine (or colon), lined by a specialized single layer of cells organized into two morphologically and functionally distinct compartments: flask-shaped submucosal invaginations known as crypts of Lieberkühn, and finger-shaped luminal protrusions termed villi, which dramatically increase the absorptive surface area of the small intestine. The crypt constitutes the proliferative compartment of the intestinal epithelium; it is monoclonal and maintained by four to six multipotent stem cells located in the lower third (Bjerknes and Cheng 1999) that give rise to intermediate descendants referred to as transit-amplifying cells. The villus represents the differentiated compartment and is polyclonal as its cells derive from several crypts (Potten and Loeffler 1990). Absorptive enterocytes, mucous-producing goblet cells, and hormone-secreting enteroendocrine cells migrate upwards along the basement membrane to the epithelium apex, where they undergo apoptosis, being subsequently exfoliated into the intestinal lumen (Hall *et al.* 1994). Paneth cells are unusual in that they settle at the crypt base and are the only cell type migrating downwards (Bry *et al.* 1994). The modular organization of the small intestine and colon is globally comparable. Histologically, there are, however, two important differences between them. The colon carries no villi; instead, it has a flat surface epithelium and larger colonic crypts extending deep into the submucosa. The relative abundance of each of the main cell types also varies markedly within the

different intestinal segments. Enterocytes are highly polarized cells responsible for absorbing and transporting nutrients across the epithelium, secreting a cocktail of hydrolytic enzymes into the gut. They make up more than 80% of all intestinal epithelial cells. Goblet cells secrete protective mucins and trefoil proteins required for the movement and effective removal of luminal contents, while providing protection against shear stress and chemical damage. Accordingly, their numbers increase from the proximal (4%) to distal (16%) intestine (Karam 1999). Enteroendocrine cells coordinate gut physiology through specific hormone production (Höcker and Wiedenmann 1998). They are scattered as individual cells throughout the mucosa, representing a small proportion (<1%) of the cells lining the epithelium (Schonhoff *et al.* 2004). Finally, Paneth cells have a function in innate immunity, synthesizing bactericidal agents such as defensins and lysozyme (Porter *et al.* 2002). They are absent from the colon and have a life expectancy of six to eight weeks (van der Flier and Clevers 2009), much longer than that of their terminally differentiated villus counterparts, with a turnover rate of roughly three to five days (Wright and Irwin 1982).

Currently, some controversy exists as to the presence of distinct types of intestinal stem cells. The "+4 position" model assumes the crypt base is exclusively populated by terminally differentiated Paneth cells and that stem cells are located just above them, on average at the +4 position (Potten *et al.* 1974). These cells were shown to divide once every day and to be unusually sensitive to radiation, possibly preventing the accumulation of deleterious genomic changes (Potten 1977). They were described to retain DNA labelling, and are also called label-retaining cells (LRCs), a feature suggested as being the result of asymmetric strand segregation (Potten *et al.* 2002). The "stem cell zone" model states that small, undifferentiated, cycling cells called crypt base columnar (CBC) cells, residing in a stem cell-permissive environment and wedged between the Paneth cells at the base of the crypts, are likely to be the true stem cells (Cheng and Leblond 1974; Bjerknes and Cheng 1999). Yet, definitive proof of stemness requires putative stem cells to be experimentally linked to their progeny, and this has proven elusive due to lack of specific markers (Barker *et al.* 2012). The musashi-1 (*MSI1*) gene encodes a RNA-binding protein initially described as a regulator of asymmetric division in neural stem cells (Glazer *et al.* 2012). Later studies showed that it denotes multipotent stem cells in other settings, being also highly expressed at the crypt base, with expression

evident on the CBC cells, as well as in some LRCs (Potten *et al.* 2003). However, its broad expression domain suggests that it is a marker of early committed progenitors alike. Lineage tracing techniques in inducible mouse models led to the identification of the leucine rich repeat-containing G-protein coupled receptor 5 (*LGR5*), the first specific CBC cell marker (Barker *et al.* 2007). The LGR5 protein acts as the receptor for a small family of Wnt agonists called R-spondins. *Lgr5*$^{+}$ cells are highly uniform in morphology, invariably touch Paneth cells, and divide each day. Another robust marker of *Lgr5*$^{+}$ stem cells called olfactomedin-4 (*OLFM4*) has recently emerged. It is a member of the olfactomedin domain-containing family and encodes a secreted glycoprotein that appears to have anti-apoptotic and cell cycle regulatory characteristics (Grover *et al.* 2010). Molecular *in situ* hybridization revealed that *OLFM4* is highly expressed in normal CBC cells in human small intestine and colon (van der Flier *et al.* 2009). The most reliable candidate as an LRC marker to date is *BMI1*, which encodes a component of the polycomb repressing complex 1 that acts as a chromatin modifier, being implicated in the stable maintenance of gene repression (Valk-Lingbeek *et al.* 2004). It has attracted attention due to its role in regulating self-renewal of neural and hematopoietic progenitors. *Bmi1* was found to mark rare cells at the +4 cell position uniquely in about 10% of the proximal small intestine (Sangiorgi and Capecchi 2008).

Modelling of the intestinal epithelium is based on a delicate balance between self-renewal and differentiation, which must be maintained throughout life. Noteworthy, in all species studied, the crypt-villus axis junction represents the physical threshold from which intestinal cells acquire their final functional characteristics, arguing for conserved molecular mechanisms involved in this process. In fact, intestinal homeostasis is dependent on autocrine and paracrine interactions between the mucosa and the underlying mesenchyme, and many of the intervening signalling pathways, such as Wnt, Bone Morphogenetic Protein (BMP), and Notch, have been identified (Crosnier *et al.* 2006). These molecular signals provide some of the basic principles through which intestinal architecture is organized, but it is still not clear at what point stem cell progeny loses its potency and becomes irreversibly committed to differentiation. Irrespectively of how this occurs, cell fate decisions need to be closely timed in relation to the pattern of cell divisions. While the mechanisms controlling intestinal cell transitions are far from completely

understood, it is obvious that they involve transcription factors conferring compartment-specific gene expression.

1.2 THE INTESTINAL TRANSCRIPTION FACTOR CDX2

When considering the molecular basis of intestinal differentiation, a common point of convergence exists, independently of the biological setting – the *caudal* (*cad*) type homeobox 2 gene (*CDX2*). This transcription factor is exclusively present in the intestine and *de novo* expressed in every foci of ectopic intestinal differentiation in the body, associated with carcinogenic processes. Its embryonic requirement and transcriptional activity over intestine-specific genes incorporate the properties of a "master regulator".

1.2.1 *CDX2* homeobox gene and its targets

One of the earliest isolated homeobox genes in *Drosophila* showed for the first time maternal as well as zygotic expression, accumulating in a concentration gradient spanning the antero-posterior axis of the embryo. During later embryogenesis, it was expressed in more posterior structures, therefore it was named *cad* (Mlodzik *et al.* 1985). Soon after, three murine orthologues designated *Cdx1* (Duprey et al. 1988), *Cdx2* (James and Kazenwadel 1991) and *Cdx4* (Gamer and Wright 1993) were characterized. With the exception of *Cdx4*, they are confined to the posterior gut endoderm during later development and the mature intestine after birth.

The human *CDX2* gene was independently cloned from an adult jejunal cDNA library (Drummond *et al.* 1997) and by differential screening of mRNA from CRC (Mallo *et al.* 1997). It maps to the ParaHox gene cluster in chromosome 13q12.3, has three exons and encodes a 313 amino acid protein containing a nuclear translocation and activation domain in the amino terminus (Trinh *et al.* 1999), and a highly conserved helix-turn-helix DNA-binding motif called the homeodomain towards the carboxyl terminus. The CDX2 homeodomain shares 100% homology with

the hamster CDX3 homeodomain, 96% with the mouse CDX2, and even 88% with the homeodomain from *Drosophila* Cad, implying recognition of similar DNA targets. CDX proteins have been demonstrated to bind as a monomer or dimer to one or more CDX-responsive elements, typically consisting of the consensus sequence (A/C)TTTAT(A/G), in direct or reverse orientation both in promoter and gene enhancer regions (Margalit *et al.* 1993; Taylor *et al.* 1997; Verzi *et al.* 2010). These motifs are frequently juxtaposing (Troelsen *et al.* 1997) or even intersecting (Lambert *et al.* 1996) a TATA-box sequence, usually part of the RNA polymerase II binding site.

Although some reports demonstrate that CDX2 can act as an indirect repressor, counteracting CDX1 or other transcription factor-mediated activation, by competing for the same binding sites (Gautier-Stein *et al.* 2003; Furumiya *et al.* 2013) or through interaction with basal components of the transcriptional machinery (Chun *et al.* 2007; Mutoh *et al.* 2010), most studies provide strong evidence of a role in activating gene transcription (Verzi *et al.* 2011). Indeed, CDX2 is determinant for the expression of numerous intestinal differentiation markers, and it has been shown to regulate absorptive lineage-specific factors such as sucrase-isomaltase (Suh *et al.* 1994; Boudreau *et al.* 2002), lactase phlorizin hydrolase (Troelsen *et al.* 1997; Fang *et al.* 2000), carbonic anhydrase 1 (Drummond *et al.* 1996), guanylyl cyclase C (Park *et al.* 2000), calbindin-D9K (Lambert *et al.* 1996; Wang *et al.* 2004a), liver-intestine cadherin (Hinoi *et al.* 2002), and villin (Yamamichi *et al.* 2009); goblet cell-specific factors such as MUC2 (Mesquita *et al.* 2003; Yamamoto *et al.* 2003) and trefoil factor 3 (Shimada *et al.* 2007); and enteroendocrine cell-specific proglucagon (Jin and Drucker 1996). On the contrary, CDX2 levels consistently seem lowest in Paneth cells, a lineage recently shown to be suppressed by CDX2 overexpression-mediated loss of nuclear β-catenin (Crissey *et al.* 2011). CDX2 is also involved in controlling the expression of additional molecules that contribute to cellular dynamics, including processes of proliferation (Uesaka *et al.* 2002), growth arrest (Bai *et al.* 2003; Aoki *et al.* 2011), migration (Coskun *et al.* 2010), adhesion (Lorentz *et al.* 1997; Sakaguchi *et al.* 2002; Hinkel *et al.* 2012), metabolism and transport (Modica *et al.* 2009; Kakizaki *et al.* 2010), inflammation (Wang *et al.* 2005), glycoproteome modulation (Isshiki *et al.* 2003), and apoptosis (Mallo *et al.* 1998). In any case, regulatory outcome ultimately results from the cooperative balance with other important transcription factors, being mostly relevant the hepatocyte nuclear factor (HNF1 and HNF4) and GATA-binding factor (GATA4/5/6) families (Boudreau *et al.* 2002; Verzi *et al.* 2010;

Verzi *et al.* 2013). On the other hand, these and other transcription factors, such as *KLF4*, can be directly regulated by CDX2 (Mahatan *et al.* 1999; Boyd *et al.* 2010). Hence, the complex hierarchies of control that govern gene expression during intestinal differentiation and development are determined by the stoichiometry of different transcription factors and cofactors within intestinal cells at any given time, as well as by the type, number, and arrangement of *cis*-acting elements in the regulatory regions of intestinal genes.

1.2.2 Role in homeostasis

During embryogenesis, CDX homologues participate in patterning of the vertebral column (Subramanian *et al.* 1995; Chawengsaksophak *et al.* 1997; van Nes *et al.* 2006), as well as in haematopoiesis (Wang *et al.* 2008), via *HOX* gene regulation and with a certain degree of functional redundancy (van den Akker *et al.* 2002; Savory *et al.* 2009). Both *Cdx1* and *Cdx2* are expressed during endoderm development and in adult intestine; however, the specific role of each member and the extent of their functional equivalence is still not completely understood. In the later embryo, CDX1 and CDX2 levels vary quantitatively along the rostrocaudal axis, with highest expression of CDX1 in the distal portion of the colon and highest CDX2 expression in the proximal colon, diminishing in either direction (James *et al.* 1994; Silberg *et al.* 2000). A fairly complementary gradient of expression has also been described along the vertical crypt-villus axis, with the former primarily localized to the crypt, and the latter primarily along the villus, although with less staining towards the tip (Silberg *et al.* 1997; Rings *et al.* 2001; Kim *et al.* 2002; Silberg *et al.* 2002). These patterns endure throughout the lifespan of the animal and are also observed in human tissue (Walters *et al.* 1997; Boulanger *et al.* 2005). They are thought to reflect intrinsically different functionalities, with CDX1 associated to a more proliferative phenotype and CDX2 to a more differentiated one, conceding a partially interchangeable activity in certain contexts (Verzi *et al.* 2011). *Cdx1*$^{-/-}$ mutants are viable and fertile, and like transgenics overexpressing *Cdx1*, display no overt intestinal phenotype (Subramanian *et al.* 1995; Bonhomme *et al.* 2008; Crissey *et al.* 2008). *Cdx2* precedes and is needed for *Cdx1* onset during intestinal development (Eda *et al.* 2002; Silberg *et al.* 2002; Mutoh *et al.* 2009), and *Cdx1* requirement is only unmasked upon *Cdx2* loss, as their combined absence in the adult stage

significantly enhances lethality effects of isolated *Cdx2* deficiency (Verzi *et al.* 2010; Verzi *et al.* 2011; Stringer *et al.* 2012). It therefore follows that CDX2 function in the adult intestine is more vital than that of CDX1.

The role of CDX2 started to be established *in vitro*, when it was observed that forced expression in the undifferentiated intestinal cell line IEC-6 arrests proliferation and initializes an epithelial polarity program (Suh and Traber 1996). *Cdx2*$^{-/-}$ mice embryos display pre-implantation lethality and die *in utero* between embryonic day 3.5 and 5.5, whereas the *Cdx2*$^{+/-}$ mutants are viable, but exhibit skeletal anomalies or stunted growth (Chawengsaksophak *et al.* 1997; Tamai *et al.* 1999). Crucial evidence that CDX2 functions in specifying intestinal cell fate was initially obtained by observing that 90% of the heterozygous mice develop multiple polyps within the first three months of life, particularly in the proximal colon. These polyps present the remaining *Cdx2* allele inactivated and reveal a homeotic reversion towards anterior differentiation (Beck *et al.* 1999). This process of intercalary regeneration means that local sporadic *Cdx2* haploinsufficiency conveys a signal for a pathway of rostral phenotype; hence, a gradient of positional information is observed, with areas of stratified squamous epithelium similar to that seen in the oesophagus, areas resembling the gastric mucosa, and even areas reminiscent of the small intestine. Wild-type male hosts with *Cdx2*$^{-/-}$ cells from mutant female donors also develop chimaeric intestinal patches of organotypically normal stomach epithelium (Beck *et al.* 2003). Of interest, the underlying host stroma concurrently assumes a gastric phenotype, proving that endodermal expression of CDX2 initiates endodermal/mesodermal cross-talk and is the primary signal for gut differentiation, subsequently involving appropriate feedback loops (Stringer *et al.* 2008). A complementary approach, relying in transgenic expression of *Cdx2* in the stomach, demonstrated that CDX2 is sufficient to induce intestinal enterocytes with enzymatic and absorptive functions *in vivo* (Mutoh *et al.* 2005b). This is reinforced by these mice being able to survive over one month after extensive small bowel resection, when compared to the short seven day lifespan of surgery-controls.

Other animal models have been independently reported, in which embryonic lethality of *Cdx2* ablation was circumvented by conditionally targeting its knockout at posterior developmental stages. Pending on temporal restrictions, this inactivation differentially impacts global intestinal morphology. Early loss results in anterior transformation of the small intestine to an oesophageal phenotype (Gao *et al.* 2009),

whereas in latter induction the intestine shows a more gastric-like identity, with impaired endo-lysosomal trafficking and inefficient segregation of apico-basal membrane domains (Gao and Kaestner 2010; Grainger *et al.* 2010). Conditional inactivation in the already adult mice compromises enterocyte function, causing severe malnutrition and death in about three weeks (Verzi *et al.* 2010; Verzi *et al.* 2011). A limited ablation strategy compatible with long-term survival has been applied in this setting revealing the presence of partial gastric-nature metaplasias associated with loss of all differentiated intestinal cell types (Hryniuk *et al.* 2012; Stringer *et al.* 2012). Hence, CDX2 stands out as being the most critical element in the development, differentiation, and maintenance of the intestinal phenotype.

1.2.3 Involvement in carcinogenesis

Beyond its homeotic function, CDX2 is also involved in processes of leukemogenesis (Lengerke and Daley 2012) and GI carcinogenesis. Conflicting data regarding the nature of CDX2 function in tumour initiation and development exist, and most probably CDX2 assumes different roles in dissimilar contexts.

IM and gastric cancer

An association between IM and intestinal-type gastric carcinoma has been previously established (Correa 1992). It is now widely recognized that the main molecular driver of this preneoplastic condition is *de novo* expression of *CDX2*. Several studies have described the presence of both CDX1 and CDX2 in nearly all gastric IM foci (Bai *et al.* 2002; Almeida *et al.* 2003; Kim *et al.* 2006; Barros *et al.* 2008). But proof of concept supporting CDX2 involvement in this process was provided by two transgenic mouse models achieving inappropriate *Cdx2* expression in the gastric epithelium. These mice develop IM, characterized by the presence of absorptive, goblet, and enteroendocrine cell-types (Mutoh *et al.* 2002; Silberg *et al.* 2002). One model even progressed to gastric cancer after long-term induction (Mutoh *et al.* 2004), reinforcing the implication of IM in the genesis of gastric carcinoma. Besides being ectopically expressed in IM, it has been repeatedly demonstrated that CDX2 expression is downregulated in the progression from IM to gastric cancer (about 50% are positive), and that CDX2 positivity significantly correlates with a more differentiated (intestinal) histology and better prognosis (Kaimaktchiev *et al.* 2004;

Mizoshita *et al.* 2004; Liu *et al.* 2007a). Nevertheless, intestinal differentiation markers are not exclusive to intestinal-type gastric cancer and the diffuse-type can also have features of intestinal differentiation (Almeida *et al.* 2003).

The spectrum of IM in the human body is broader. Accordingly, CDX2 has been shown to be present in abnormal foci of intestinal differentiation in the oesophagus, liver, gallbladder and pancreas (Barros *et al.* 2012). These metaplasias also bear clinical significance. For example, CDX2 is ectopically expressed in Barrett's oesophagus, a precancerous condition that develops from mucosal injury incurred after chronic gastroesophageal acid and bile reflux, harbouring an increased risk of oesophageal adenocarcinoma development (Eda *et al.* 2003; Groisman *et al.* 2004; Yousef *et al.* 2008). Aberrant expression of CDX2 is also detected in IM of the gallbladder, a lesion associated with cholelithiasis, dysplasia, and carcinoma (Wu *et al.* 2005; Sakamoto *et al.* 2007).

Colorectal cancer

The role of CDX2 protein during CRC development remains controversial, as different studies suggest both negative and positive modulation of tumourigenesis. In this regard, *Cdx2*$^{+/-}$ heterozygous mice develop polyp-like lesions characterized by gastric heteroplasia in the colon, as described above (Chawengsaksophak *et al.* 1997; Beck *et al.* 1999; Tamai *et al.* 1999). These are more sensitive to azoxymethane-induced colonic adenocarcinoma than wild-type mice (Bonhomme *et al.* 2003). Similarly, it was shown that the colonic polyp number is about six times higher in *Apc*$^{+/\Delta 716}$ *Cdx2*$^{+/-}$ compound mutant mice compared to heterozygous *Apc*$^{+/-}$ littermates, which mainly develop adenomatous polyposis of the small intestine (Aoki *et al.* 2003). CDX2 expression has also been reported to be markedly reduced at later stages of human colorectal carcinogenesis (Mallo *et al.* 1997), particularly in high grade dysplasia and during adenocarcinoma invasion (Ee *et al.* 1995). Furthermore, exogenous CDX2 expression in human colon cancer cell lines induces a less malignant phenotype, inhibiting proliferation and invasion, while promoting the expression of genes characteristic of mature intestinal cells (Suh and Traber 1996; Mallo *et al.* 1998; Gross *et al.* 2008). Together, the above findings suggest that CDX2 functions as a putative tumour-suppressor and this has been the prevailing paradigm in this context. Conversely, CDX2 expression seems to be heterogeneously lost at the invasive tumour edge (Brabletz *et al.* 2004) and only reduced in a subset of CRCs,

particularly those with minimal differentiation (Hinoi *et al.* 2001; Hinoi *et al.* 2003). In agreement, loss of chromosome 13 has been seldom observed in CRCs and *CDX2* sequence mutations are exceedingly rare, only occurring in repeat sites of MSI$^+$ cancers (Wicking *et al.* 1998; da Costa *et al.* 1999; Woodford-Richens *et al.* 2001; Subtil *et al.* 2007). It was also reported that CDX2 overexpression in colon cancer cell lines can induce anchorage-independent growth and migration (Uesaka *et al.* 2002; Dang *et al.* 2006; Chun *et al.* 2007). Moreover, immunohistochemical analysis has commonly detected strong CDX2 expression in more than 80% of human colon cancer samples (Moskaluk *et al.* 2003; Werling *et al.* 2003; Kaimaktchiev *et al.* 2004; Witek *et al.* 2005). Recently, recurrent 13q12 chromosomal amplifications targeting the *CDX2* locus were described in CRCs (Salari *et al.* 2012). It was demonstrated that colorectal-derived tumours and cell lines with amplification exhibit CDX2 dependency concerning cell survival and proliferation, leading to its classification as a lineage-survival oncogene.

1.2.4 CDX2 regulatory network

The low rate of *CDX2* genomic alterations supports the notion that regulation, as opposed to structural mutation, accounts for altered CDX2 levels and

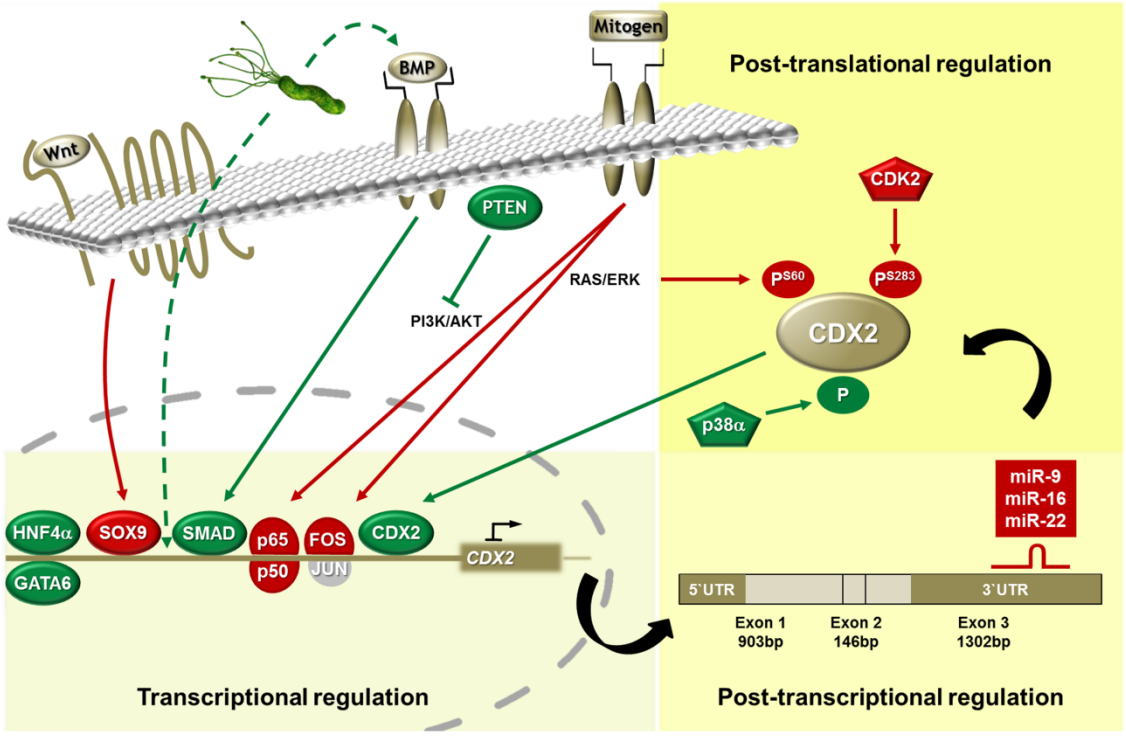


Figure 2. CDX2 regulatory network. Schematic representation of the main mechanisms of CDX2 regulation at the gene, transcript and protein level. Green elements are general activators of expression or activity, while red elements depict general repressors.

consequently, for maintaining its normal expression pattern and function under homeostasis. Indeed, several transcriptional, post-transcriptional and post-translational mechanisms have been described to control CDX2, making this a highly complex and tightly organized regulatory system (**Figure 2**).

Transcriptional regulation

The distinct *Cdx2* expression patterns observed embryonically and during postnatal life seem to be driven by different genomic fragments. Indeed, the *Cdx2* promoter contains intestine-specific regulatory elements that interact with and respond to combinations of different transcription factors integrated within particular signalling pathways.

Wnt/β-catenin pathway

The Wnt pathway plays a crucial role in demarcating the intestinal as opposed to gastric fate in the embryonic endoderm, as assessed by the expression of intestinal and gastric genes (Kim *et al.* 2005). A synergistic effect over the *Cdx2* promoter is obtained with HNF4α and GATA6 expression, which is enhanced by the presence of TCF4 and β-catenin (Benahmed *et al.* 2008). The relevance of this combination is underlined by being able to activate ectopic *CDX2* expression in non-intestinal HeLa cells. However, the effect of the Wnt pathway is intricate, because besides its stimulatory role during development, it indirectly downregulates *CDX2* via the intestine crypt transcription factor SOX9 (Blache *et al.* 2004). In contrast, *CDX2* mRNA expression was found to be upregulated by APC (da Costa *et al.* 1999), which in turn can be induced by CDX2 (Lorentz *et al.* 1997; Olsen *et al.* 2013). This inhibits cell proliferation, probably by disrupting the β-catenin/TCF protein complex and its transcriptional activity (Guo *et al.* 2010). These studies argue for the existence of positive and negative feedback loops between the Wnt/β-catenin pathway and CDX2, whose aftermath may depend upon the cellular and tissue context.

BMP pathway

In line with the crucial role of BMPs in intestinal differentiation, and with the demonstration that there is an influx of BMP2- and BMP4-producing inflammatory cells to the stomach upon *H. pylori* infection (Bleuming *et al.* 2006), the BMP pathway was shown to be active in gastric IM (Barros *et al.* 2008). BMP4 was also found in the stromal tissue underlying inflamed oesophageal squamous epithelium and Barrett's

metaplasia (Milano *et al.* 2007). In addition, BMP4 treatment of human primary cultured oesophageal cells was reported to induce columnar intestinal-type differentiation, accompanied by *de novo* CDX2 expression, which might be relevant for the development of Barrett's oesophagus (Zhou *et al.* 2009). Another natural occurring model supporting the role of this pathway in CDX2 regulation was provided by the observation that loss of BMP activity in juvenile polyps correlates with loss of intestinal differentiation and the appearance of gastric metaplasia (Barros *et al.* 2009b). *Helicobacter* spp. infection was recently shown to upregulate the BMP pathway in the gastric context, concomitantly mediating CDX2 increased and SOX2 decreased expression (Camilo *et al.* 2012). In the human digestive tract, SOX2 shows an inversely correlated expression pattern to CDX2, decreasing and gradually disappearing as IM progresses from the incomplete to the complete type, making it a putative gastric differentiation factor (Tsukamoto *et al.* 2004; Asonuma *et al.* 2009). Accordingly, SOX2 was shown to counteract the effect of HNF4α and GATA6 over the activation of the *Cdx2* promoter (Benahmed *et al.* 2008).

Ras/MAPK pathway

Elevated ERK1/2 activities stimulate S phase entry of intestinal cells and promote proliferation in response to mitogenic stimulation (Aliaga *et al.* 1999). Conversely, low sustained levels of the same pathway act as a convergent signal towards G1 arrest and intestinal differentiation (Taupin and Podolsky 1999). Accordingly, activation of ERK1/2 by oncogenic *RAS* in human colonic cancer cells modifies the JUN/FOS balance favouring a FOS negative effect over the *Cdx2* promoter (Lorentz *et al.* 1999; Krueger *et al.* 2009).

PI3K/AKT pathway

PTEN-mediated tumour suppressive function is invariably linked to PI3K signalling inhibition, and it is not surprising to find PTEN decreased expression concomitant with inappropriate activation of PI3K pathway as one of the most frequently observed features in many human cancers, including colorectal (Zhang *et al.* 2011). It was shown that PTEN induces *Cdx2* expression by inhibiting the PI3K/AKT pathway, via differential binding activity of the p50 and p65 NF-κB subunits at the *Cdx2* promoter, with preferential allocation of the p50/p50 homodimer (Kim *et al.* 2002). PTEN activity also promotes intestinal differentiation of gastric cancer cells by increasing *CDX2* expression (Semba *et al.* 2009). The expression levels of *CDX2* are significantly

decreased and inversely correlated with the expression pattern of TNF-α during active stages of inflammatory bowel disease and in a mouse model of dextran sulphate sodium-induced colitis (Coskun *et al.* 2012). Treating human colon cancer cells with TNF-α, an activator of the PI3K signalling pathway, has a contrary effect to the one described for PTEN, by preferentially stimulating the DNA binding activity of the NF-κB p65/p50 heterodimer at the *Cdx2* promoter, decreasing its expression and transcriptional activity (Kim *et al.* 2002).

Autoregulation

The observation that *Cdx2*$^{+/-}$ mice do not express the remaining wild-type allele in developing intestinal polyps, without evidence for loss of heterozygosity, led to postulate that *Cdx2* expression could be regulated, in part, by a self-regulatory mechanism. The first hints on this possibility were provided by transactivation assays performed in pancreatic and intestinal cell lines showing that CDX2 could positively regulate its expression in a cell type-specific manner (da Costa *et al.* 1999; Xu *et al.* 1999). These results were confirmed and further extended in relevant biological settings provided by the mouse intestine and human IM, where CDX2 was shown to bind to its own promoter, which in the latter case, might have an impact in the stability of the phenotype and carcinogenic progression (Barros *et al.* 2011).

Epigenetic modifications

Methylation is a biochemical modification that consists in the addition of a methyl group to cytosine residues predominantly, but not exclusively, at CpG dinucleotides enriched within so-called CpG islands. These often overlap functional elements, for example, gene promoter regions. It has been shown that exposure to acid and/or biliary salts may activate *CDX2* expression in human oesophageal epithelial cells through promoter demethylation (Liu *et al.* 2007b). Dietary habits could be an important factor determining the methylation status and expression levels of *CDX2* in gastric carcinogenesis, as epidemiological studies in gastric cancer patients established an inverse correlation between *CDX2* methylation and selective lifestyle factors, like green tea and vegetable intake (Yuasa *et al.* 2005; Yuasa *et al.* 2009). Aberrant *CDX2* methylation was frequently observed in CRCs (Kawai *et al.* 2005), although other study reported that this was a rare event and described it as a unique property of squamous oesophageal cancer cells (Guo *et al.* 2007). DNA methylation and histone modifications associated with *CDX1* and *CDX2* promoters

were also analysed in two human colon cancer cell lines and reported to be heterogeneous, but at least for *CDX2*, differential expression was not due to methylation (Lu *et al.* 2008).

Other environmental influences

In the setting of gastric carcinogenesis, *H. pylori* infection is undoubtedly considered a major starting point for malignancy. In this regard, several studies showed isolated cells or foci of reduced CDX2 expression, mainly in the antral gastric epithelium, without any histological evidence of IM in chronic *H. pylori* infected individuals (Satoh *et al.* 2002; Bornschein *et al.* 2009). This pattern of expression was referred to as "positive staining of single cells" and considered an early, albeit still reversible indication of the gastric mucosa intestinalization, strongly related with *H. pylori* infection and gastritis (Vauhkonen *et al.* 2008). Ensuing, *H. pylori* infection was shown to directly exert a positive regulatory effect over *CDX2* in gastric cancer cell lines (Matsuda *et al.* 2008; Barros *et al.* 2009a). *Helicobacter* spp. infection can also focally induce *de novo* CDX2 expression in the stomach of mice, in a context of mild inflammation and without signs of morphological changes resembling IM (Camilo *et al.* 2012). Nevertheless, the underlying molecular mechanisms linking *H. pylori* presence and CDX2 expression have just started to be revealed, and the BMP/SMAD pathway seems to be a determinant mediator of the bacteria-induced alterations in the gastric epithelial gene expression program.

In the oesophageal context, chronic exposure of murine keratinocytes to either acid (Marchetti *et al.* 2003) or bile acids (Kazumori *et al.* 2006) was shown to induce *Cdx2* transcription, an effect also confirmed in human oesophageal epithelial cells by acid/bile salts combinatorial treatment (Debruyne *et al.* 2006; Zhou *et al.* 2009; Huo *et al.* 2010). Since Barrett`s development is clinically associated with gastroesophageal reflux and inflammatory cell infiltration, stable changes in the pH balance might constitute a key trigger in the early pathological intestinal differentiation process of oesophageal cells. Curiously, in gastric metaplasia of the duodenum, a lesion characterized by the inverse transformation process of intestinal epithelial cells into gastric foveolar cells, as a result of increased gastric acid load, transcription of *Cdx2* and its intestinal-target genes is impaired (Faller *et al.* 2004). These studies point to the role of the chemical microenvironment as a preponderant factor involved in the regulation of tissue-specific gene expression programs.

As for the influence of stromal elements, it was observed early on that epithelial-mesenchymal cellular interactions can differentially affect the expression of CDX1 and CDX2 homeobox genes (Duluc *et al.* 1997). For example, different components of the basement membrane matrix, which lie at the interface of and are synthesized by epithelial and mesenchymal cells, such as Laminin-α1, can positively modulate CDX2 expression (Lorentz *et al.* 1997). A stimulation of CDX2 expression by subepithelial colonic myofibroblasts was also reported, mediated by the non-canonical Wnt family member WNT5A and the epithelial receptor ROR2, contributing to inhibition of the canonical Wnt signalling and intestinal differentiation (Pacheco and Macleod 2008). On the other hand, collagen type I induces phenotypic changes in CRC cells through the β1-integrin/FAK signalling pathway that involve reduced *Cdx2* promoter activity and mRNA expression (Brabletz *et al.* 2004). In agreement with this, a model of orthotopic and heterotopic xenografs in nude mice demonstrated that CDX2 expression is adaptable and strongly dependent on the microenvironment (Benahmed *et al.* 2007).

Post-transcriptional regulation by microRNAs

Due to the absence of CDX2 expression in about half of the gastric cancer cases and heterogeneous loss in CRCs, it was hypothesized that microRNAs (miRs) could also be associated with CDX2 silencing in these contexts. Computational prediction of miR-binding sites, using independent databases, led to the selection of miR-9 and miR-204 as putative candidates for CDX2 regulation (Rotkrua *et al.* 2011). A comparative analysis of CDX2 and miR-9 expression in a panel of gastric cancer tissues revealed an overall inverse correlation between both, and *in vitro* experimental data, confirmed that miR-9 interacts directly and specifically with the *CDX2* 3`untranslated region (UTR), leading to downregulation of CDX2 target genes and promotion of cell growth. More recently, it was demonstrated that exogenous CDX1-induced expression of miR-9, miR-16 and miR-22 suppresses *CDX2* mRNA by targeting its 3`UTR in a CRC cell line (Tagawa *et al.* 2012).

Post-translational regulation

Phosphorylated forms of p38 MAPKs were shown to be mostly retained in the nuclei of villus cells, with differential p38α kinase activity constituting an early and necessary event for the initiation of the intestinal differentiation program (Houde *et*

al. 2001). This stimulatory effect is accomplished by CDX2 phosphorylation, enhancing its transcriptional ability without involvement in the loss of proliferative potential or cell survival. On the other hand, phosphorylation by ERK1/2 MAPKs of serine 60 within the amino-terminal activation domain of CDX2, reduces its transcriptional capability and impairs certain intestinal differentiation properties (Rings *et al.* 2001; Lemieux *et al.* 2011). This modification was mainly found in the proliferating compartment of the intestine, while the nonphosphorylated and more active CDX2 was predominantly found in the differentiated region, in agreement with the localization of pERK1/2 (Aliaga *et al.* 1999). In addition, activation of the ERK1/2 MAPK cascade was shown to promote ubiquitin-proteasome-dependent turnover of the CDX2 protein (Krueger *et al.* 2009). Two independent studies have elucidated how CDX2 regulation can be coordinated with the cell cycle machinery. It was observed that CDX2 undergoes CRM1-dependent nuclear export and subsequent proteolytic degradation by interaction with CDK2 in proliferative intestinal cells (Boulanger *et al.* 2005). The CDK2-mediated phosphorylation of CDX2 was found to occur downstream of the homeodomain at serine 283, identified as being part of a conserved motif of four evenly spaced serines called the 4S motif, similar to the one controlling β-catenin degradation by the proteasome (Gross *et al.* 2005). Preventing phosphorylation through this site blocked polyubiquitination and stabilized CDX2, with impact on overall cell behaviour. In conclusion, CDX2 contains multiple phosphorylation sites that either positively or negatively balance its activity and/or stability in response to different signalling pathways, being conceivable that their combined action is required for the onset of the complete differentiation process.

1.3 POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

Presently, it is well established that gene expression is regulated at multiple levels, as previously demonstrated for CDX2, and that the diverse processes involved are integrated within each other. Transcriptional control is one of the most important steps within the gene regulation cascade. Nevertheless, the significance of post-

transcriptional control is evolving, as the ability to reprogram protein synthesis is a common theme in embryonic development, wound healing, inflammation, metabolic stress and aging. An overview of the main concepts of post-transcriptional regulation is presented, focusing on RNA-binding proteins (RBPs) and their roles in development and disease.

1.3.1 From transcripts to proteins

An increasing number of publications show that a poor correlation between steady-state transcript abundances and corresponding protein pools generally exists in almost every organism (de Sousa Abreu *et al.* 2009; Maier *et al.* 2009; Vogel *et al.* 2010). In accordance, a recent study comprehensively analysed mRNA and protein levels, half-lives, transcription and translation rate constants for thousands of genes in NIH3T3 mouse fibroblasts, and found that mRNA levels only explain around 40% of the variability in protein levels (Schwanhäusser *et al.* 2011). Whether this exact percentage is valid for other cell types is unknown. Thus, it is clear that a significant fraction of Eukaryotic gene regulation is post-transcriptional in nature.

Post-transcriptional regulation encompasses RNA processing, localization, translation and decay, as well as RNA stability throughout. These interconnected mechanisms provide complementary quality-control layers that collectively define the fate of every transcript (Moore 2005). Adding to the complexity, RNAs do not dwell alone in the cell, as they are ever accompanied by *trans*-acting factors, namely RBPs and non-coding RNAs, such as miRs, that bind *cis*-elements usually present in the 3´UTR (Kuersten and Goodwin 2003; Huntzinger and Izaurralde 2011). These are capable of adjusting the amount of gene product more rapidly, precisely and with a tactical reversibility option that transcriptional regulation alone cannot offer. It is this unique escort, their relative positions, and interactions that create a highly dynamic web of messenger ribonucleoprotein (mRNP) complexes, ruling RNA life. Indeed, Eukaryotic mRNPs have been functionally considered "post-transcriptional operons" that markedly expand the regulatory elasticity of our surprisingly small genomes (Keene and Tenenbaum 2002; Keene 2007). Evidence in favour of this enticing RNA operon model on a genome-wide scale is mounting, as studies show that discrete subsets of mRNAs with shared sequence elements and

encoding proteins with common functions and locations are coordinately regulated by specific mRNPs (Gerber *et al.* 2004; Hogan *et al.* 2008).

RNA processing starts in the nucleus, where precursor mRNAs acquire a 7-methylguanosine cap structure at the 5' terminus (Lewis and Izaurralde 1997), a specialized poly(A) tail at the 3` end (Mangus *et al.* 2003), and introns are removed by splicing (Matlin *et al.* 2005). A major remodelling in mRNP composition occurs as mature mRNAs pass through the nuclear-pore complex (Köhler and Hurt 2007), with some factors remaining stably associated, whereas others are dynamically replaced by cytoplasmic counterparts. Upon export, several mRNAs are integrated in the translationally active pool, a process that remodels the protein coat and assembles polysomes. The canonical mechanism of translation is intricate and involves three coordinated events: initiation, elongation and termination (Jackson *et al.* 2010). On the other hand, some mRNAs are programmed for delayed translation, which allows transcripts to be transported to a specific subcellular localization or even stored until developmental or environmental cues call for their protein synthesis. These mRNAs are packaged into cytoplasmic mRNP granules that lack a limiting membrane and are visible under light microscopy, called processing bodies (P bodies) and stress granules (Anderson and Kedersha 2006).

1.3.2 RNA-binding proteins and their biological functions

An extensive computational analysis established that RBPs comprise 8 to 15% of the protein coding repertoire in eukaryotic genomes, highlighting both their ancient origin and the importance of RNA regulation in cell function (Anantharaman *et al.* 2002). One possible explanation is that as highly specific processes to fine-tune gene expression evolved, a concomitant expansion of the number of RBPs needed has occurred. For example, at least 74% of human genes express multiple isoforms by using different exonic combinations through alternative splicing (Johnson *et al.* 2003). Its emergence during evolution drove the need for a corresponding increase in the number of RBPs, and it is also, in itself, a mechanism by which cells can expand their RBP repertoire.

A subset of these proteins recognizes common features to almost every message, such as the 5`cap or the 3`poly (A) tail. However, the majority have a requirement for a primary sequence or type of secondary structure in which the

former is embedded, that are present in some mRNAs but not others. Their specificity is mediated by unique structural arrangements of individual RNA-binding domains whose properties are further moderated by auxiliary domains. This property, coined as "cooperative modularity", accommodates different functionalities and allows for enormous combinatorial potential, further increasing affinity towards RNA (Lunde *et al.* 2007). To date, more than 40 different binding domains have been proposed, being the most frequent the RNA recognition motif. Other common classes include the K-homology (KH) domain, zinc-fingers of the CCCH and CCHC type, and the double-stranded RNA-binding domain.

RBPs capacity to virtually regulate every aspect of RNA biogenesis and function is remarkable. In fact, they play pivotal roles during embryonic development (Kuersten and Goodwin 2003), and in a breath of several homeostatic processes such as synaptic plasticity (Luo *et al.* 2010), immune responses (Anderson 2008), epithelial cell proliferation (Yang *et al.* 2011), differentiation (Yang *et al.* 2010) and polarity (Nagaoka *et al.* 2012). With increasing knowledge on their importance, the more apparent it becomes that tampering with RBPs expression or function underlies the onset of several pathological conditions, including cancer (Lukong *et al.* 2008). Members of the signal transduction and activation of RNA (STAR) family of RBPs play vital roles in cell proliferation and differentiation. SAM68, for instance, is overexpressed in breast (Lukong *et al.* 2005) and prostate cancer cells (Busà *et al.* 2007). On the contrary, Quaking (QKI) seems to function as a tumour suppressor in CRCs (Yang *et al.* 2010) and glioblastomas (Chen *et al.* 2012).

1.4 THE MEX-3 FAMILY OF RNA-BINDING PROTEINS

Members of an evolutionarily-conserved family of RBPs, the MEX-3 family, are emerging as important post-transcriptional regulators of several cellular processes in diverse physiological settings. Their functions and underlying molecular mechanisms, particularly well-known for the ancestral *mex-3* in the model organism *Caenorhabditis elegans*, are now described.

1.4.1 *Caenorhabditis elegans* MEX-3

In the worm embryo, normal development requires precise spatial and temporal expression patterns of maternal mRNAs, such as the anteriorly localized membrane receptor *glp-1* and the posteriorly localized transcription factor *pal-1*, the *Drosophila notch* and *cad* orthologues, respectively (Evans and Hunter 2005). The correct distribution of these messages that act to direct lineage-specific commitment patterns from individual blastomeres, is determined by partitioning-defective (*par*) genes, which are required to establish polarity and whose disruption cause the earliest and most extensive embryonic abnormalities (Kemphues *et al.* 1988; Goldstein and Macara 2007). Molecular evidence places *C. elegans* MEX-3 amid both classes, an intermediate regulator of cell fate determinants under *par*-dependent control, thus acting as a critical component in the link between cell polarity and asymmetric gene expression (Huang *et al.* 2002).

mex-3 gene encodes a protein with two seventy-amino acid regions that are 40% identical to each other. The repeated sequences correspond to putative RNA-binding motifs, initially identified in the pre-mRNA-binding heterogeneous nuclear ribonucleoprotein (hnRNP) K and named KH domains (Siomi *et al.* 1993). MEX-3 interacts with a minimal MEX-3 recognition element (MRE), defined as the degenerate consensus sequence (A/G/U)(G/U)AG$N_{(0-8)}$U(U/A/C)UA (Pagano *et al.* 2009). Mutations disrupting the *mex-3* locus are fully penetrant, recessive, strict maternal-effect and embryonically lethal, resulting in embryos that abnormally generate body-wall muscle from the anterior blastomere (AB), hence the name *mex* for "*muscle excess*" (Draper *et al.* 1996). This homeotic transformation is due, in part, to the function MEX-3 exerts over the transcription factor *pal-1*, the orthologue of *CDX* genes in mammals, and the somatic determinant of posterior identity (Hunter and Kenyon 1996). *mex-3* mRNA and protein accumulate cytoplasmically and are unevenly distributed in early embryos (Draper *et al.* 1996). After fertilization and between the two and four-cell stage, MEX-3 is preferentially localized to the AB lineage, disappearing afterwards in a pattern similar to that described for other maternal mRNAs (Seydoux and Fire 1994). On the other hand, while *pal-1* mRNA is present throughout the early embryo, PAL-1 protein is asymmetrically localized and only detected from the four-cell stage onwards in descendants of the posterior (P1) blastomere lineage, thus correlating with low MEX-3 levels (**Figure 3A**). In accordance, PAL-1 is ectopically expressed in all cells of *mex-3* mutant embryos and

MEX-3 has been shown to repress translation of a *lacZ* RNA reporter construct containing the *pal-1* 3`UTR in anterior blastomeres (Hunter and Kenyon 1996). Therefore, MEX-3 plays a crucial role as a translational repressor of *pal-1*, specifying proper blastomere identity during early embryogenesis.

Post-transcriptional regulation of maternal RNAs is also a primary mechanism to control gene expression in *C. elegans* germline, and MEX-3 contributes to the maintenance of germ cell totipotency in the adult worm (Ciosk *et al.* 2006). The hermaphrodite germline is a highly dynamic organ presenting a stereotypical

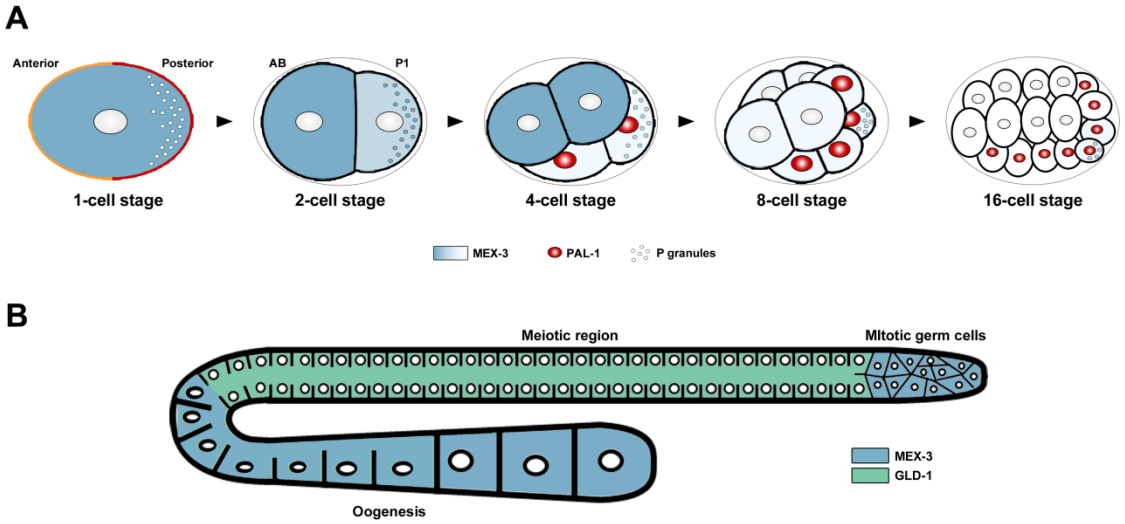


Figure 3. MEX-3 protein distribution in *Caenorhabditis elegans* embryo and adult germline. (**A**) Representation of the inversely correlated MEX-3 and PAL-1 proteins expression pattern from 1- to 16-cell stage. MEX-3 is also involved in the correct segregation of P granules to the final germline precursor. (**B**) Diagram of a wild-type gonad arm showing MEX-3 and GLD-1 proteins mutually exclusive configuration along the sequential assembly of germ cells, progressing through mitosis, meiosis, and oogenesis.

organization of gametes commitment: the most distal end of the gonad is a proliferative compartment containing mitotic cells that progressively enter meiosis in a central syncytial region. Spontaneous germ cell death by apoptosis or differentiation into sperm cells (late L4 larval stage) and oocytes (adulthood) occurs in the proximal gonad. Switches between these different stages involve spatially non-overlapping translational regulation of target mRNAs by the RBPs defective in germline development (GLD-1) and MEX-3 (Lee and Schedl 2001; Mootz *et al.* 2004) (**Figure 3B**). In maturing oocytes, MEX-3 also mediates *pal-1* inhibition; combined with GLD-1 action, this ensures that embryos do not inherit maternal PAL-1 protein and develop properly (Draper *et al.* 1996; Mootz *et al.* 2004). In accordance, double mutant strains for *mex-3* and *gld-1* develop a germline tumour containing numerous cells of muscular, neuronal and intestinal nature, reminiscent of human

teratomas (Ciosk *et al.* 2006). This germ cell transdifferentiation seems to be the end result of an abnormal expression of somatic determinants such as PAL-1.

1.4.2 The mammalian MEX3 proteins

C. elegans mex-3 was identified and characterized in humans as a family of four homologous genes called *MEX3A* to *MEX3D* (Buchet-Poyau *et al.* 2007). These are located at chromosomic positions 1q22, 15q25.2, 18q21.2 and 19p13.3, respectively, and are composed of two exons and one intron. The closely related encoded proteins contain two tandemly repeated KH domains that putatively provide RNA-binding properties. Additionally, they possess a carboxy-terminal really interesting new gene (RING) finger module, not present in the nematode MEX-3 and believed to mediate protein-protein interactions. Four mouse orthologues were also identified displaying strong similarity at the amino acid level to their human counterparts. For these characteristics they are also known as RING finger and KH domain-containing (RKHD) proteins.

Expression analysis of the *MEX3* transcripts in different human tissues showed that *MEX3D* is ubiquitous, while the others have a varied expression pattern, with the highest level found in fetal brain and testis (Buchet-Poyau *et al.* 2007). Overexpression experiments demonstrated that MEX3 are phosphoproteins that shuttle between the nucleus and the cytoplasm by the CRM1-dependent export pathway and via an N-terminally located export signal. Confocal microscopy analysis revealed co-localization of MEX3A and MEX3B with DCP1A and AGO family members, being the latter catalytic components of the RNA-induced silencing complex (RISC), the key effector of miR and RNA interference (RNAi) pathways. This association was restricted to P bodies, which are known sites of mRNA turnover (Eulalio *et al.* 2007). These results together with the ability of MEX3 proteins to interact *in vitro* with poly(A) ribonucleotide homopolymers and certain cellular mRNAs, indicate that MEX3 proteins constitute novel human RBPs potentially involved in post-transcriptional regulatory networks (Donnini *et al.* 2004; Buchet-Poyau *et al.* 2007; Courchet *et al.* 2008). Nevertheless, the specific biological role of each member was basically unknown during the course of this work, and only recently started to be explored in more detail.

1.5 LONG-TERM GOAL AND SPECIFIC AIMS

The homeobox transcription factor CDX2 is determinant for proper embryonic development, as well as for endodermal induction and adult maintenance of the intestinal epithelium. Besides this homeostatic function, *in vitro* experiments, animal models and human lesions show that *de novo* expression of CDX2 is associated with ectopic foci of intestinal differentiation, which in turn conveys an increased risk of cancer development. A paradigmatic example of this link is provided by gastric IM, a lesion that encompasses a tissue adaptive response to *H. pylori* infection and subsequent chronic inflammatory reaction. The role of bacterial eradication as a common preventive strategy against gastric cancer development remains debatable and largely dependent on the extent of preneoplastic changes at the time of treatment, given the apparent stability of the CDX2-dependent IM phenotype. Moreover, the nature of CDX2 involvement in the establishment and/or progression of CRC is far from being consensual. Early data suggested that loss of CDX2 was a common event; however, ensuing studies have shown that its expression is retained and in some cases even increased. Therefore, defining a thorough portrayal of the CDX2 regulatory network stands out as a leading investigation priority, not only to advance its specific contribution to these carcinogenic processes, but most importantly to apply the acquired knowledge in the design of alternative and perhaps more target-oriented therapeutic strategies.

The present work, framed within the abovementioned conceptions, intends to uncover new molecular mechanisms of CDX2 regulation focusing on the role of yet poorly-described microenvironmental influences as a research guiding line. In this regard, the following specific objectives are proposed:

1. Evaluate if methylation at the promoter level is a mechanism involved in the regulation of *CDX2* gene expression

DNA methylation is an epigenetic mechanism of transcriptional regulation and a direct measure of the environmental effect over the genome, with an involvement in cancer attributed to the inappropriate silencing of tumour suppressor genes (hypermethylation), or loss of oncogene repression (hypomethylation).

Previous studies reported that the methylation status of *CDX2* might have a relevant regulatory role in different contexts. But they present discrepancies, both concerning the region of query and the methods employed to analyse it. The lack of

a comprehensive analysis led us to evaluate if methylation at the promoter level could be a mechanism involved in the regulation of CDX2 expression, and the obtained results are presented in Chapter 2. We hypothesized that in intestine, where CDX2 is expressed, the gene would be unmethylated, while in the normal gastric mucosa, where CDX2 is not detected, the gene would be methylated. The process of *H. pylori* infection and accompanying inflammatory response would in some way be responsible for promoter demethylation, constituting the triggering event leading to the development of IM. The alteration in *CDX2* methylation profile along the gastric carcinogenic process would constitute a proof of concept, undoubtedly establishing the relevance of this mechanism and contributing to settle a contentious question in the field.

2. Assess the role of cell-matrix interactions in the regulation of CDX2 expression

For many years now, traditional methods of cell culture have produced important conceptual advances in cancer research. Nevertheless, cells grown on flat substrates can differ considerably in their morphology, differentiation, cell-cell and cell-matrix interactions from those growing in more physiological conditions. At the other end of the spectrum we have whole-animal models, which frequently corroborate the importance of particular processes. But these are complex, expensive and not easily manipulated. By mimicking features of the *in vivo* microenvironment and taking advantage of the same tools used to study cells in standard culture, 3D cell/tissue models have been bridging the gap between both, providing unique perspectives on the behaviour of stem cells, epithelial tissues and tumours.

Earlier publications described the role of epithelial-mesenchymal interactions as determinant for the expression of CDX2, not only for proper spatio-temporal induction of intestinal differentiation, but also in modulating its levels during carcinogenesis. However, the direct molecular mediators responsible for this adaptable response remained for the most part elusive. In Chapter 3, we present results regarding the establishment of *in vitro* 3D cell culture models comprising gastric cancer cell lines and an extracellular matrix. We hypothesized that this approach, exploratory in nature, would allow pinpointing regulatory factors and signalling pathways involved in the control of CDX2 expression.

3. Determine if the RNA-binding protein MEX3A retains a repressive function over CDX2

Post-transcriptional mechanisms are now considered at least as equally important as transcriptional ones for the life of the cell, adding further complexity to gene regulatory networks. The modulation of the cell culture microenvironment achieved in the previous chapter, fused with high-throughput genome-wide screening, led to the identification of a target putatively involved in CDX2 regulation, called mex-3 homologue A (*MEX3A*).

In *C. elegans*, MEX-3 plays a crucial role as a translational repressor of *pal-1*, the *CDX* orthologue, specifying blastomere identity during embryogenesis. Although the mammalian MEX3 family members had been shown to interact *in vitro* with RNAs, their biological role was essentially unknown. We hypothesized that the RNA-binding protein MEX3A could have an evolutionarily conserved role in inhibiting CDX2 expression, this case in the GI context. In Chapter 3, we challenge this idea by performing diverse cell-based assays together with expression studies in murine intestine, with the objective of unravelling mechanistic and functional aspects behind such regulation. In Chapter 4, we highlight recent scientific advancements on the function of the mammalian MEX3 proteins, comparing common aspects of biological relevance, discussing their putative importance in development and disease, and also raising future points of interest in this research area.

Chapter 2

CDX2 promoter methylation

The results concerning this chapter are published in:

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Letter to the Editor

CDX2 promoter methylation is not associated with mRNA expression

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Dear Sir,

Yuasa *et al.*[1] published that specific lifestyle factors, described to be potentially preventive of gastric cancer on epidemiological basis, may directly influence the carcinogenic process by affecting demethylation or maintenance of unmethylated status of selected genes. Specifically, it was shown that *CDX2* was methylated in 23.6% of primary gastric carcinomas, whereas no methylation was seen in adjacent noncancerous gastric tissue of some of the cancer patients studied. They also demonstrated a significant inverse association between intake of green tea (more than 7 cups/day) and the methylation status of CDX2 in gastric cancer patients. However, in the same article, the authors did not show that the methylation status was correlated with CDX2 expression. In our study, we investigated if methylation of the CDX2 promoter could be a mechanism involved in the regulation of CDX2 expression in gastric carcinomas using gastric carcinoma cell lines as models and also in intestinal metaplasia (IM) of the stomach. CDX2 is a transcription factor that plays a major role in intestinal differentiation both in the intestine and in aberrant locations, such as in IM of the stomach.[2,3] We show that there is no association between *CDX2* promoter methylation and CDX2 expression, suggesting that methylation does not constitute a primary mechanism regulating CDX2 expression both in gastric carcinoma cell lines and in gastric preneoplastic lesions. Thus, our findings do not support the conclusions taken by Yuasa *et al.*[1]

We used the bioinformatic tool CpGPlot to search for potential CpG islands in the proximal region of the *CDX2* promoter and identified two regions that fulfil the criteria (Fig. 1*a*). Then we studied the basal CDX2 mRNA expression and the methylation status of the *CDX2* promoter in a panel of four human gastric carcinoma cell lines (AGS, GP202, IPA220 and MKN45). CDX2 mRNA expression analysis in the gastric carcinoma cell lines showed that AGS and IPA220 strongly express CDX2, while GP202 and MKN45 present equally low levels of CDX2 expression (Fig. 1*b*). The methylation of the *CDX2* promoter in these cell lines was determined using the bisulfite-genome sequencing method (Fig. 1*c*). CpG island 1 was methylated in all cell lines, whereas CpG island 2 was methylated in GP202 and AGS cell lines and unmethylated in IPA220 and MKN45 cell lines. These results did not correlate with CDX2 mRNA expression. To further confirm if methylation could be in any way involved in *CDX2* regulation, we treated these cell lines with the demethylating agent 5-aza-2'-deoxycytidine (Fig. 1*d*). Once more, the results obtained suggest that methylation is not directly regulating CDX2 expression since cell lines with similar basal methylation status, AGS and GP202, react differently to the treatment with a demethylating agent. Although there is an increase of expression in GP202 after treatment, CDX2 remains unchanged in AGS. Strikingly, MKN45 which shows unmethylated *CDX2* promoter also reacts to the treatment with an increased expression.

Furthermore, we examined CDX2 expression and the methylation status of 42 CpG sites, contained in CpG island 2 of the *CDX2* promoter, in two specimens of normal gastric mucosa, adjacent IM foci and in two normal colonic mucosa samples. As expected no CDX2 expression was observed in normal gastric mucosa in contrast to IM and colonic tissue (data not shown). The methylation pattern was inconsistent and no correlation was found with CDX2 expression in all samples (Fig. 2).

In summary, and although previous studies show that methylation might be involved in *CDX2* regulation,[4–7] our *in vitro* and *in vivo* results demonstrate that the pattern of methylation at the *CDX2* promoter is random and unrelated with its expression. The increase in CDX2 expression observed in some of the cell lines studied is more likely due to the effect of the demethylating agent over epigenetically modified regulators, such as silenced transcription factors or modified histones,[8] and not directly over *CDX2*.

Yours sincerely,

Bruno PEREIRA, Carla OLIVEIRA, Leonor DAVID, Raquel ALMEIDA*

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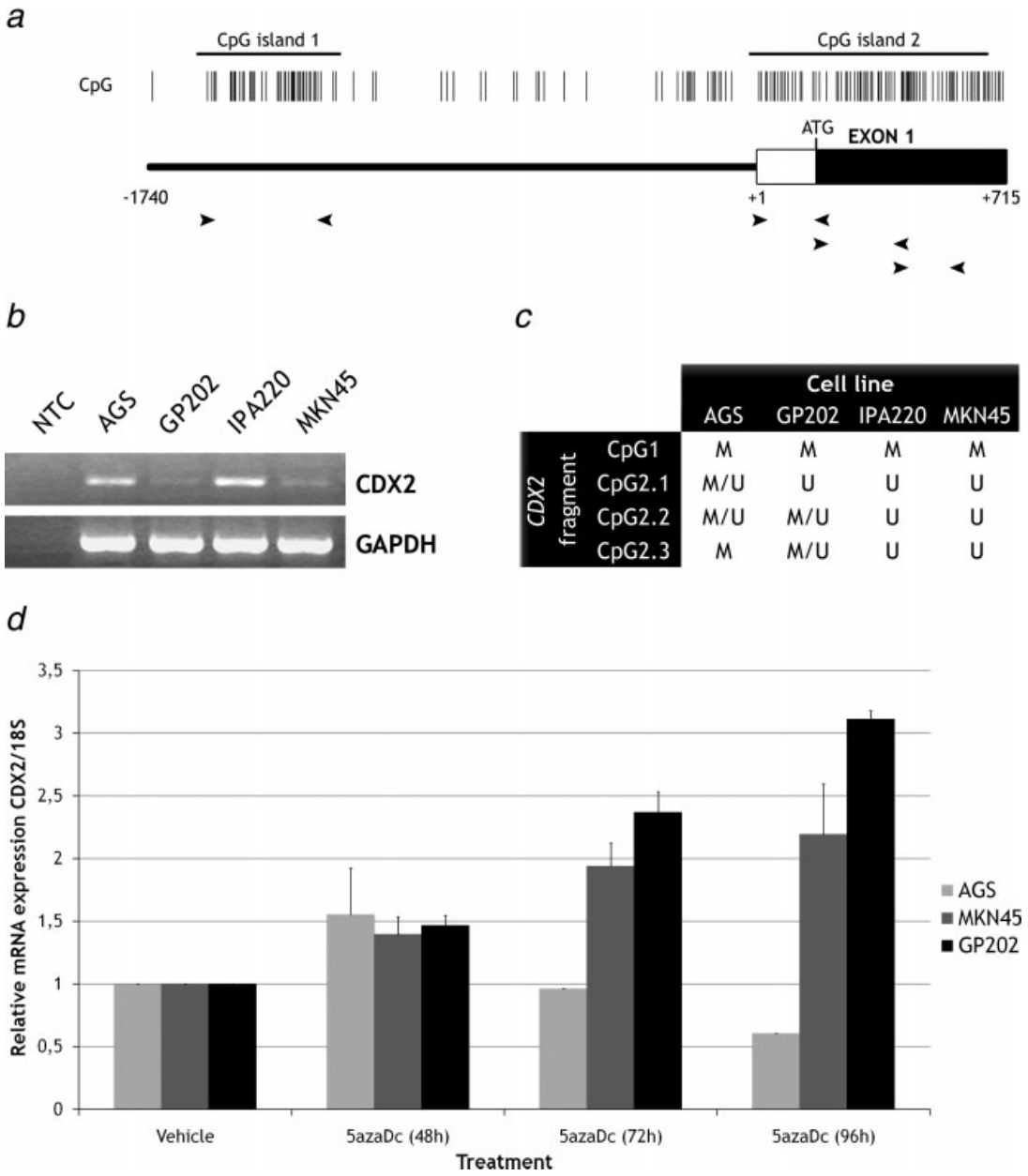


FIGURE 1 – Methylation status of *CDX2* in a panel of human gastric cancer cell lines and functional relation with *CDX2* mRNA expression. (*a*) Schematic representation of the 5`proximal flanking region of *CDX2* gene. Thin vertical lines mark the location of CpG dinucleotides. A box indicates the first exon, including non-coding (white) and coding (black) sequences. Two putative CpG islands were identified with the online bioinformatic tool CpGPlot (www.ebi.ac.uk/tools/emboss/cpgplot), one in the promoter region (–1603bp to –1197bp) and another in the first exon, including the coding region (–22bp to +660bp). Arrow-heads indicate the position of bisulfite specific primers. (*b*) RT-PCR analysis of *CDX2* mRNA expression in gastric cancer cell lines. *GAPDH* mRNA expression was used as an internal loading control and NTC indicates no template control. (*c*) Methylation profile of *CDX2* in gastric cancer cell lines obtained by direct sequencing of bisulfite-treated genomic DNA. M, methylated; U, unmethylated. (*d*) Real-time PCR for *CDX2* expression in human gastric cancer cell lines after treatment with the demethylating agent 5-aza-2`-deoxycytidine (5azaDc, Sigma) during different time-points. Each experiment was carried out in triplicate at least twice; the results are expressed as mean ± SD of biological replicas. The values obtained with vehicle-treated cells were referred to as 1. *CDX2* mRNA levels were normalized with the corresponding *18S* rRNA levels.

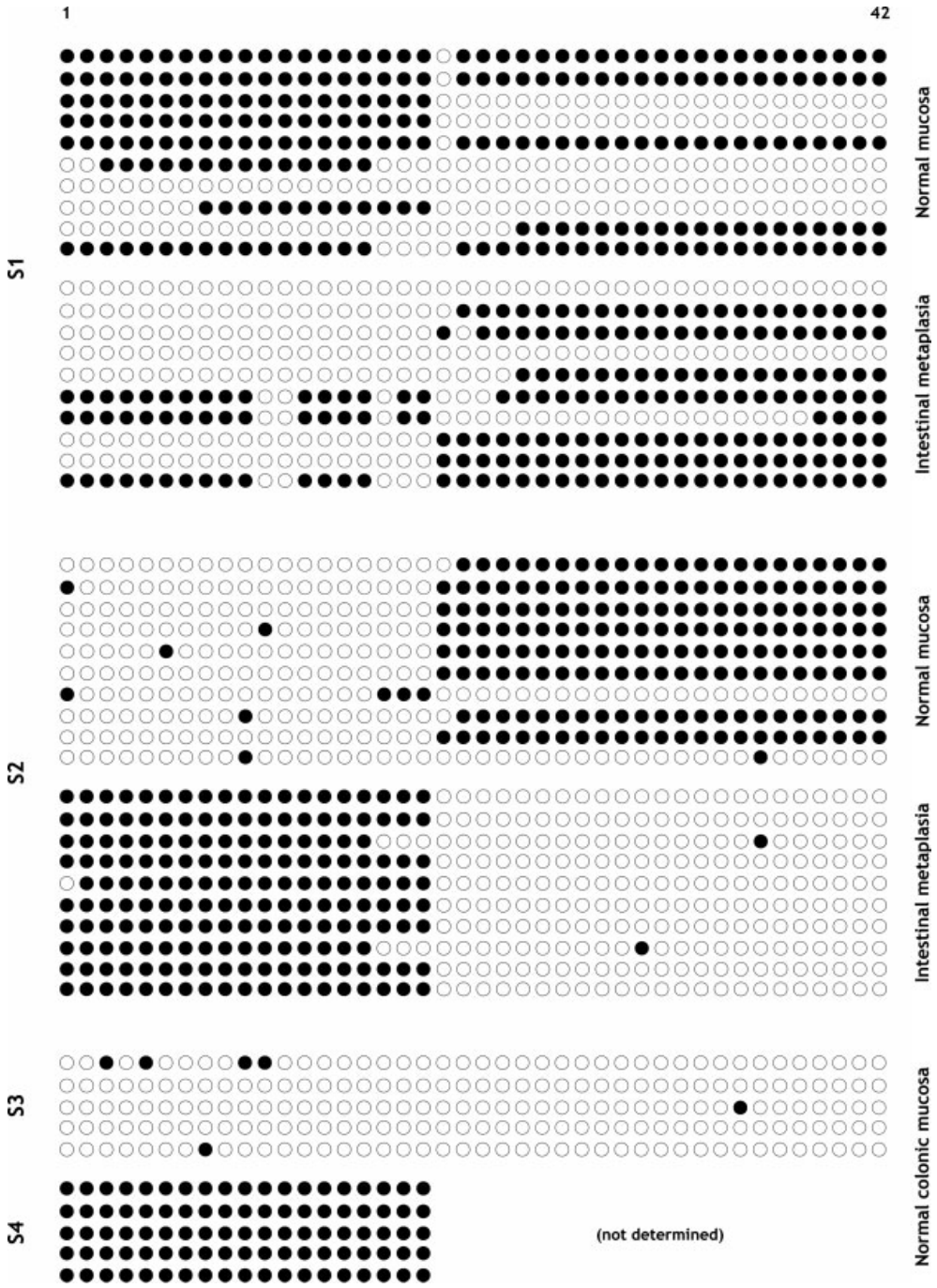


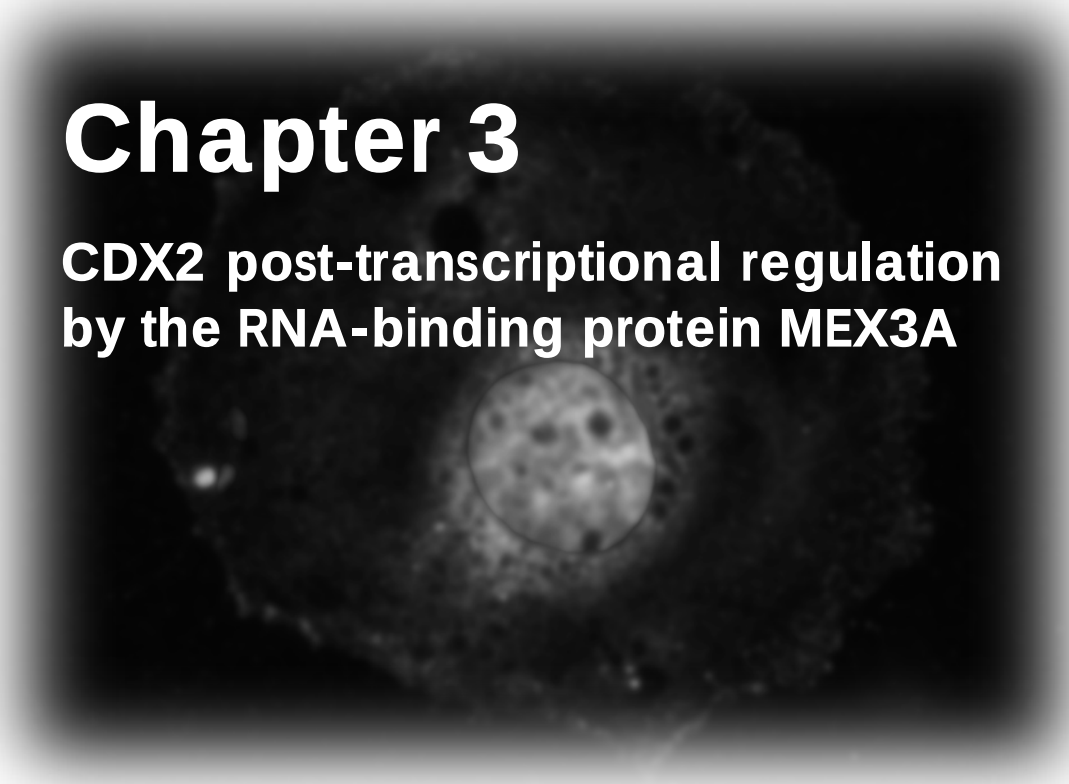
Figure 2 – Bisulfite DNA sequencing of the first and second fragments of *CDX2* CpG island 2 in two specimens of the gastric mucosa and adjacent IM foci (S1 and S2) and in two specimens of normal colonic mucosa (S3 and S4). Only clearly identifiable normal gastric glands and metaplastic glands were selected using a PALM Microbeam Microscope (Zeiss). With this methodology we were able to minimize stromal cell contamination in our tissue samples. PCR amplicons from the tissues were cloned into pCR4-TOPO vector using the TOPO TA Cloning kit (Invitrogen), and at least five individual clones from each sample were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with a reverse M13 primer. Each horizontal row of circles represents analysis in a single clone of bisulfite-treated DNA of the 42 CpG sites contained in the mentioned region. Solid and open circles represent methylated and unmethylated CpG sites, respectively.

References

1. Yuasa Y, Nagasaki H, Akiyama Y, Hashimoto Y, Takizawa T, Kojima K, Kawano T, Sugihara K, Imai K, Nakachi K. DNA methylation status is inversely correlated with green tea intake and physical activity in gastric cancer patients. Int J Cancer 2009;124:2677–82.
2. Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang J, De Bolbs C, David L. Expression of intestine-specific transcription factors. CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. J Pathol 2003;199:36–40.
3. Mesquita P, Raquel A, Nuno L, Reis CA, Silva LF, Serpa J, van Seuningen I, Barros H, David L. Metaplasia—a transdifferentiation process that facilitates cancer development: the model of gastric intestinal metaplasia. Crit Rev Oncog 2006;12:3–26.
4. Guo M, House MG, Suzuki H, Ye Y, Brock MV, Lu F, Liu Z, Rustgi AK, Herman JG. Epigenetic silencing of CDX2 is a feature of squamous esophageal cancer. Int J Cancer 2007;121:1219–26.
5. Yuasa Y, Nagasaki H, Akiyama Y, Sakai H, Nakajima T, Ohkura Y, Takizawa T, Koike M, Tani M, Iwai T, Sugihara K, Imai K, et al. Relationship between CDX2 gene methylation and dietary factors in gastric cancer patients. Carcinogenesis 2005;26:193–200.
6. Kawai H, Tomii K, Toyooka S, Yano M, Murakami M, Tsukuda K, Shimizu N. Promoter methylation downregulates CDX2 expression in colorectal carcinomas. Oncol Rep 2005;13:547–51.
7. Liu T, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS, Chen X. Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. Carcinogenesis 2007;28:488–96.
8. Fujii S, Ochiai A. Enhancer of zeste homolog 2 downregulates E-cadherin by mediating histone H3 methylation in gastric cancer cells. Cancer Sci 2008;99:738–46.

Chapter 3

CDX2 post-transcriptional regulation by the RNA-binding protein MEX3A



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The author declares that he performed and/or was involved in all the experimental work, participated in the study design and wrote the manuscript. Microarray experiments were conducted at NFDM (National Facility for DNA Microarrays, Universidade de Aveiro, Portugal) and 3D cell cyst assays at Institut Albert Bonniot (INSERM-UJF U823, Grenoble, France).

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CDX2 regulation by the RNA-binding protein MEX3A: impact on intestinal differentiation and stemness

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ABSTRACT

The homeobox transcription factor CDX2 plays a crucial role in intestinal cell fate specification, both during normal development and in tumorigenic processes involving intestinal reprogramming. The CDX2 regulatory network is intricate, but it has not yet been fully uncovered. Through genome-wide screening of a 3D culture system, the RNA-binding protein MEX3A was identified as putatively involved in CDX2 regulation; therefore, its biological relevance was addressed by setting up cell-based assays together with expression studies in murine intestine. We demonstrate here that MEX3A has a repressive function by controlling CDX2 levels in gastric and colorectal cellular models. This is dependent on the interaction with a specific binding determinant present in *CDX2* mRNA 3`untranslated region. We have further determined that MEX3A impairs intestinal differentiation and cellular polarization, affects cell cycle progression and promotes increased expression of intestinal stem cell markers, namely *LGR5*, *BMI1* and MSI1. Finally, we show that MEX3A is expressed in mouse intestine, supporting an *in vivo* context for interaction with CDX2 and modulation of stem cell properties. Therefore, we describe a novel CDX2 post-transcriptional regulatory mechanism, through the RNA-binding protein MEX3A, with a major impact in intestinal differentiation, polarity and stemness, likely contributing to intestinal homeostasis and carcinogenesis.

INTRODUCTION

The homeodomain transcription factor CDX2 is a critical determinant of intestinal homeostasis, both during development and throughout adult life. CDX2 is involved in the antero-posterior patterning of the mammalian embryo and is the key molecular mediator of intestinal differentiation (1–4). Furthermore, multiple evidences substantiate CDX2 crucial role in carcinogenesis of the digestive tract. It was shown to inhibit cell growth and migration *in vitro* as well as dissemination of colon tumour cells *in vivo* (5). CDX2 heterogeneous loss has also been observed in colorectal carcinomas (CRCs), particularly in invasive cells at the tumour edge (6). Moreover, CDX2 reduction increases the progression of chemically induced CRCs (7). Conversely, under certain pathological conditions, CDX2 becomes abnormally expressed in other organs of the digestive tract besides intestine, namely the esophagus

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(8) and stomach (9,10), driving a precancerous lesion known as intestinal metaplasia, a process confirmed in transgenic mouse models (11,12).

Owing to the essential function in intestinal development, differentiation and carcinogenesis, CDX2 regulation has been extensively studied. We have previously identified different mechanisms involved in the transcriptional regulation of this gene such as the Bone morphogenetic protein (BMP) pathway (13), SOX2 (14) and a CDX2 autoregulatory loop (15). Several transcription factors including HNF4a, GATA6, TCF4 and β-catenin were shown to interact with *Cdx2* promoter fragments (16). However, mutations at the *CDX2* locus are a rare event in CRC (17), and its expression does not depend on methylation of the proximal promoter (18). On the other hand, CDX2 protein phosphorylation has also been shown to modify its activity in intestinal cells (19,20). These and other studies support the notion that CDX2 regulation is intricate and strictly controlled.

During the past two decades, post-transcriptional regulation emerged as a fundamental mechanism guiding gene expression in higher eukaryotic cells, being at the core of normal cellular processes but also cancer initiation and development. It is now increasingly clear that RNA maturation, localization, translation and stability provide multiple layers of spatio-temporal control determining a transcript's fate (21,22). These coupled events are generally dependent on the cooperation between *cis*-regulatory elements and *trans*-acting factors, such as non-coding RNAs and RNA-binding proteins (RBPs). RBPs have been implicated in virtually every aspect of RNA metabolism (22,23), particularly, their repressive role is critical to establish precise translational patterns that define developmental and differentiation switches in many organisms.

In *Caenorhabditis elegans*, MEX-3 is a translational repressor that regulates blastomere identity during early embryogenesis (24) and germline totipotency in the adult worm (25). MEX-3 has two K homology domains, which are conserved single-stranded RNA-binding motifs (26). Mutations disrupting *mex-3* locus are lethal, resulting in embryos that inappropriately generate body-wall muscle from the anterior blastomere; hence, the name *mex* for 'muscle excess'. This is specified, in part, by the repressive function that MEX-3 exerts over the transcription factor *pal-1*, the *C. elegans* orthologue of *caudal* in *Drosophila* and *CDX* in mammals (24,27). In humans, *mex-3* was identified and characterized as four homologous genes, *MEX3A–D* (28,29), whose biological relevance is starting to be explored. Recently, the functional role of MEX3C as a RNA-binding ubiquitin E3 ligase was established, mediating the post-transcriptional decay of HLA-A allotypes (30). It was also shown to be necessary for normal postnatal growth in mutant mice by enhancing the local expression of insulin-like growth factor 1 in bone (31) and appears to be involved in metabolic regulation of energy balance (32), through yet unknown effectors. A variant form of MEX3D called TINO was shown to negatively regulate the antiapoptotic protein BCL-2 in HeLa cells (33). Finally, knockdown of MEX3A by siRNAs was shown to suppress cell proliferation and migration in human gastric cancer cells, but the molecular mechanisms behind these findings were not addressed (34). Pursuing the aim of uncovering new CDX2 regulatory mechanisms, we explored a putative translational repression by MEX3A, which was inversely correlated with CDX2 in a 3D experimental model. By studying MEX3A expression *in vivo* and using a cell line-based approach to modulate its levels *in vitro*, our study describes a novel post-transcriptional process by which CDX2 expression is impaired in the gastrointestinal setting and intestinal-like homeostasis compromised, through alterations in differentiation, polarity and stemness features. Another layer of control is thus added to the complex CDX2 regulatory network, involving MEX3A as a key regulator of intestinal homeostasis, which might have significant implications to gastrointestinal carcinogenesis.

MATERIALS AND METHODS

Cell culture and treatments

Human gastric carcinoma cell line AGS (ATCC, American Type Culture Collection) and CRC cell line Caco-2 (ATCC) were cultured under standard conditions in RPMI-1640 medium and Dulbecco's modified Eagle's medium, respectively, containing 10% fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Life Technologies). For the AGS 3D culture, flasks were coated with 50 µl/cm^2 of matrigel basement membrane (BD Biosciences) at a 1.5:1 proportion to serum-free medium. A 2 x 10^4 cells/cm^2 suspension was seeded on top and maintained for 14 days with medium change every 2 days. For the Caco-2 3D culture, coverslips were coated with 60 µl/cm^2 of matrigel. A 6 x 10^3 cells/cm^2 suspension plus 2% matrigel was seeded on top and maintained for 8 days with medium change every 2 days. To quantify lumen formation, >100 cysts were microscopically examined. For proteasome inhibition, cells were treated with 25 µM MG132 (Calbiochem) or vehicle treated with DMSO. To inhibit transcription, cells were exposed to 10 µg/ml of Actinomycin D (Sigma).

Constructs and site-directed mutagenesis

The previously published pCMV-MEX3A expression vector (28) was used together with a pCMV-Tag3B empty vector (Agilent Technologies) in transfections. A pRLControl construct containing a humanized *Renilla* luciferase (*Rluc*) coding sequence (35) was used as a backbone to create the pRLCDX2 vector, encoding a luciferase fusion transcript to the parental *CDX2* 3`untranslated region (UTR). The QuickChange site-directed mutagenesis kit (Stratagene) was then used to introduce specific mutations in the previous plasmid, to generate the pRLΔCDX2 construct, with a mutated MEX-3 recognition element (MRE). Oligonucleotides containing the desired mutations were designed according to the manufacturer's instructions (Supplementary Table S1). Taking into account the degenerate consensus sequence described for MEX-3 binding in *C. elegans*, a mutational background of Cytidine was used because presumably this base is not tolerated in the MRE (36).

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Transfections, RNAi and luciferase assays

Transient transfections were done using Lipofectamine 2000 reagent according to the manufacturer's guidelines (Life Technologies). A DNA (μg) to Lipofectamine 2000 reagent (μl) ratio of 1:1.5 in OPTI-MEM medium was used for routine transfection experiments of expression vectors. For stable transfections, selection was initiated 48h post-transfection in medium supplemented with 0.6 mg/ml of G418 (Sigma). Neomycin-resistant positive clones obtained through limiting dilution were routinely maintained with 0.2 mg/ml of G418. A commercial set of three Stealth Select siRNA duplexes (HSS150674, HSS150675 and HSS150676) directed against human *MEX3A* (Life Technologies) and a custom set of three siRNA duplexes directed against *CDX2* were used with scrambled controls. An siRNA duplexes (pmol) to Lipofectamine 2000 reagent (μl) ratio of 20:1 in OPTI-MEM medium was used for inhibition experiments. Luciferase reporter assays were performed with the *Rluc* Assay System (Promega), and β-galactosidase activity was used for normalization of experimental variations.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using TRI Reagent (Sigma) and reverse transcribed using the Superscript II Reverse Transcriptase kit (Life Technologies). Analysis of *BMI1*, *CDX2*, *GAPDH*, *LGR5*, *MEX3A* and *Rluc* mRNA expression was performed in an ABI Prism 7500 system using SYBRgreen reagent (Life Technologies) and specific primer pairs (Supplementary Table S1). Each sample was amplified in triplicate and specificity confirmed by dissociation analysis. The *18S* rRNA expression was measured for normalization of target gene abundance.

Microarrays and data processing

Experiments were performed at the National Facility for DNA Microarrays (Aveiro, Portugal). Three independent 2D and 3D AGS cell cultures were selected for total RNA extraction using TRI Reagent. RNA quantity and quality were assessed using the Nanodrop ND-1000 (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies) systems, and only samples with a RNA integrity number above nine were considered for further study. Preparation and labelling of the RNA was performed using the One-Color Microarray-Based Gene Expression Analysis Quick Amp Labelling kit (Agilent Technologies). Briefly, 600 ng of total RNA from 2D and 3D biological replicas was used as input together with spike-in controls to generate Cyanine 3-labelled cRNA. The amplified cRNA samples were purified with RNeasy Mini kit (Qiagen) and hybridized to Human Gene expression 4 x 44K v2 Microarray slides (Agilent Technologies) at 65ºC for 17h. After washing procedures, images of the hybridizations were acquired using a G2505B Microarray Scanner (Agilent Technologies). The Feature Extraction software was used for spot identification, background subtraction and quantification of the fluorescent signal. Raw expression data were processed using BRB-ArrayTools v3.8.1 software (37). After base 2 log transformation and average of probe replicates, normalization was applied using the median intensity over the entire array to minimize systematic variance. Differentially expressed transcripts between the two culture conditions were filtered using the Class Comparison tool, performing an unpaired sample *t*-test with a $P < 0.01$ and considering a minimal 1.5-fold change. Hierarchical clustering analysis of significantly altered genes was conducted in TIGR MultiExperiment Viewer v4.8.1 software (38) using Euclidean correlation and average linkage clustering, and expression values indicated colorimetrically. Functional annotation of differentially expressed genes, identified by extending the unpaired *t*-test parameter to $P < 0.05$ and maintaining the minimal fold change was performed with DAVID program (39). Microarray data have been submitted to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) and assigned the identifier E-MTAB-1234. The complete list of differentially expressed genes is detailed in Supplementary Table S2.

Protein extraction and western blot analysis

Cells were lysed for 30 min on ice in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid and 1% IGEPAL (Sigma), supplemented with Complete protease inhibitor cocktail (Roche Applied Science), 1 mM PMSF and 1 mM Na_3VO_4. Lysates were centrifuged at 12 000 rpm for 20 min at 4ºC and the supernatant recovered. Protein concentration was determined using the BCA Protein Assay Reagent (Thermo Scientific). Protein extracts (30–50 μg) were run on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, blotted overnight with appropriate antibodies (Supplementary Table S1), and signals revealed with ECL detection kit (GE Healthcare Life Sciences). Actin levels were used to normalize protein expression, and quantification of western blots was performed using Fiji software (40).

Flow cytometry

Caco-2 cells were harvested 48 h after transfection at confluence and fixed with 1% paraformaldehyde for 20 min at room temperature followed by permeabilization with 0.1% Triton X-100 (Sigma) for 5 min on ice. Staining was performed with anti-MEX3A antibody and visualized with goat anti-rabbit FITC-conjugated secondary antibody. For DNA content assessment, cells were incubated with a propidium iodide (50 μg/ml) and RNAse A (200 μg/ml) solution (Sigma) for 30 min at room temperature. Samples were read in a FACSCanto II (BD Biosciences) flow cytometer, and analysis was performed using the FlowJo software.

RNA-immunoprecipitation assay

Cells were lysed 48 h after transfection as indicated for protein extraction but with the addition of 20 U/ml of RNAseOUT Ribonuclease inhibitor (Life Technologies). Before the lysis procedure, cells were washed with phosphate buffered saline (PBS) and subjected to ultraviolet crosslink (254-nm wavelength) at an energy level

of 1.8 J/cm^2 on ice for 20 min. Immunoprecipitation from protein extracts (750 µg) was performed overnight with anti-myc antibody (Clontech) or normal mouse immunoglobulin G (IgG) (Millipore) and RNA-immunocomplexes recovered by incubation with protein G-sepharose bead slurry (Sigma) for 2 h in the following day. After five washes in lysis buffer containing RNAseOUT, RNA was extracted from the bead slurry as indicated before and resuspended in 10 ml of dH_2O. RT-quantitative real-time PCR (qPCR) analysis was directly performed on the RNA as described. Target mRNA levels immunoprecipitated in the MEX3A transfected cells were normalized to the levels recovered in the empty vector transfected cells.

Immunofluorescence and microscopy

Cells grown on coverslips were fixed using ice-cold methanol for 10 min. For Caco-2 3D assay, cysts were fixed with a 2% paraformaldehyde and 0.05% glutaraldehyde solution at room temperature for 20 min, followed by quenching with 0.1% $NaBH_4$ and permeabilization with 0.5% Triton X-100. Paraffin-embedded wild-type murine tissue sections (4 mm) were deparaffinized with clear-rite (Thermo Scientific) and rehydrated. All samples were blocked with non-immune serum diluted in 10% BSA–PBS, followed by incubation with appropriate antibodies (Supplementary Table S1) diluted in 5% BSA–PBS. Nuclei were counterstained with 4`,6-diamidino-2-phenylindole (DAPI, Sigma), and coverslips mounted on microscope slides with Vectashield (Vector). Imaging acquisition was based on optical sectioning and adjusted for brightness and contrast with Fiji software (40). Imaging was performed on an Axio Imager Z1 (Zeiss) Fluorescence microscope with an ApoTome attachment (Figures 3, 7 and 8 and Supplementary Figure S4), and on a TCS SP5 II (Leica) Laser Scanning Confocal microscope (Supplementary Figure S1). The middle focal plane of Z-stack images with similar total number of planes were used for representation of cysts polarity.

Statistics

Each experiment was carried out in triplicates at least three times, and data were expressed as means ± SD. Statistical analysis was performed using Student's *t*-test. A $P < 0.05$ was considered as significantly different.

RESULTS

CDX2 expression is downregulated in AGS cells cultured in matrigel

In an effort to study CDX2 regulation in a physiologically relevant microenvironment, we compared CDX2

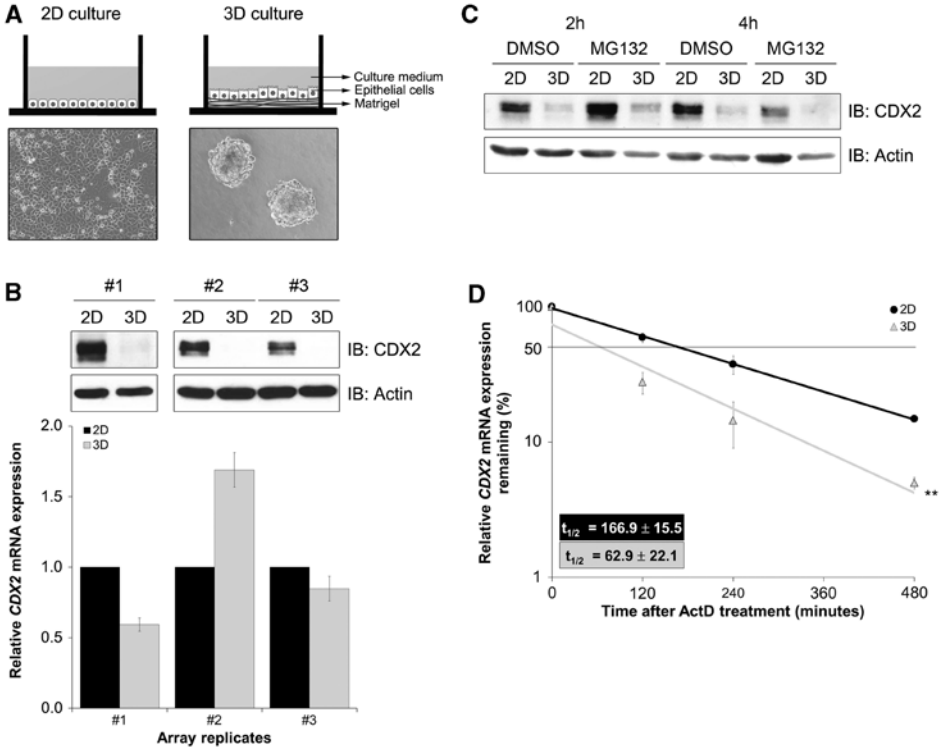

Figure 1. Establishment of AGS 3D cultures and characterization of CDX2 expression. (**A**) Schematic representation of the culture systems and cellular morphology at culture day 4 in bright field microscopy (original magnification, x100). (**B**) Western blot and qPCR of CDX2 expression in 2D and 3D after 2 weeks culture. Cardinal numbers represent biological replicates. Values for *CDX2* mRNA expression in 2D culture were referred to as 1. (**C**) Western blot of CDX2 protein expression on treatment with MG132. (**D**) qPCR of *CDX2* mRNA expression on treatment with actinomycin D. Expression levels in the absence of treatment were set at 100%. Depicted half lives were calculated using exponential regression (**$P = 0.003$).

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expression in AGS cells cultured in a standard 2D condition versus a 3D condition with matrigel. Cells depicted distinct growth patterns amongst both settings, forming a compact monolayer and conspicuous multicellular aggregates, respectively (Figure 1A). Although there was a dramatic decrease in CDX2 protein levels in the 3D culture, mRNA showed heterogeneous levels without correlation with protein (Figure 1B). Treating cells with the proteasome inhibitor MG132 indicated that CDX2 downregulation was not related to an increase in proteasome-dependent degradation (Figure 1C). On the other hand, we found a significant difference in *CDX2* mRNA stability after actinomycin D treatment to inhibit transcription (Figure 1D). The *CDX2* mRNA half-life value was higher for the 2D culture (166.9 ± 15.5 min) than for the 3D culture (62.9 ± 22.1 min). These results suggest the existence of a post-transcriptional regulatory mechanism, probably acting over *CDX2* mRNA.

With the objective of pinpointing the mechanism underlying CDX2 downregulation, we performed a whole-genome expression array, comparing the transcriptome of AGS cells in the two culture conditions. Among the 340 differentially expressed transcripts, a 1.7-fold upregulation of MEX3A was observed in the 3D condition (Figure 2A and Supplementary Table S2). Additionally, functional annotation analysis showed alterations related to fundamental cellular processes like proliferation, differentiation and survival, namely an increase in cell cycle elements, Notch and Wnt pathways and a decrease in MAPK and Jak-STAT signalling in the 3D model (Figure 2B). An increase in RhoGTPases and integrin signalling was also registered, supporting the occurrence of increased cell-matrix interactions. Changes in metabolic patterns were evident by an increase in amino acid biosynthesis (Figure 2B). MEX3A is one of the human orthologues of MEX-3, an RBP with translational inhibitory function known to target the *CDX* orthologue *pal-1* during *C. elegans* embryonic development. A novel interaction between human MEX3A and CDX2 in a gastrointestinal context could constitute a plausible explanation for the reduction of CDX2 protein levels observed in 3D culture. Accordingly, *MEX3A* expression was validated by qPCR (Figure 2C).

MEX3A overexpression leads to CDX2 downregulation in AGS cells

To define whether CDX2 expression is regulated by MEX3A, AGS cells cultured in standard conditions were transiently transfected with a myc-tagged MEX3A expression vector and collected 24 and 48 h later. We showed that the myc-tagged MEX3A fusion protein was detectable on transfection and accompanied by a 77 and 64% decrease in CDX2 protein expression, respectively (Figure 3A). In contrast, *CDX2* mRNA expression did not change (Figure 3B). This result was confirmed in AGS cells stably transfected with the myc-tagged MEX3A construct up until 96 h of culture (Figure 3C and D).

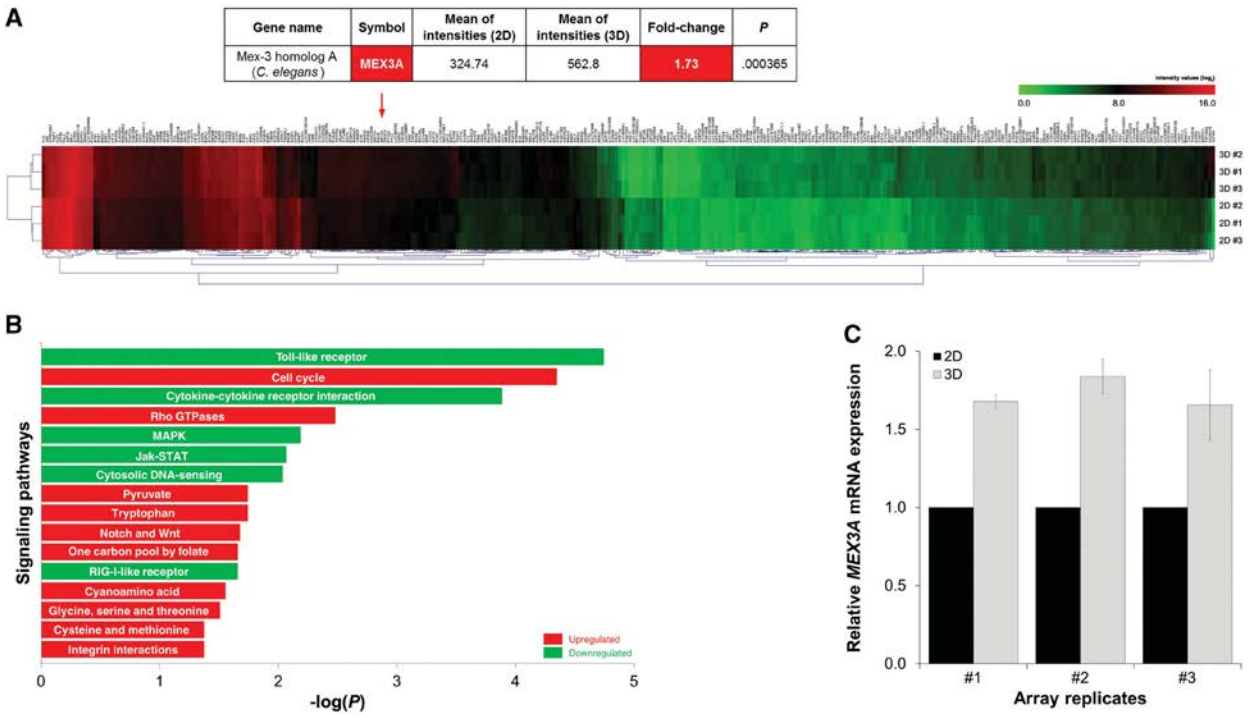

Figure 2. Transcriptome profiling of 2D and 3D AGS cultures. (**A**) Microarray heat map displaying differentially expressed transcripts ($P < 0.01$, fold change > 1.5). Values obtained for *MEX3A* are highlighted. (**B**) Functional annotation analysis depicting the preponderance of differentially expressed genes in terms of signaling networks ($P < 0.05$, fold change > 1.5). (**C**) qPCR validation of *MEX3A* expression. Values for *MEX3A* mRNA expression in 2D culture were referred to as 1.

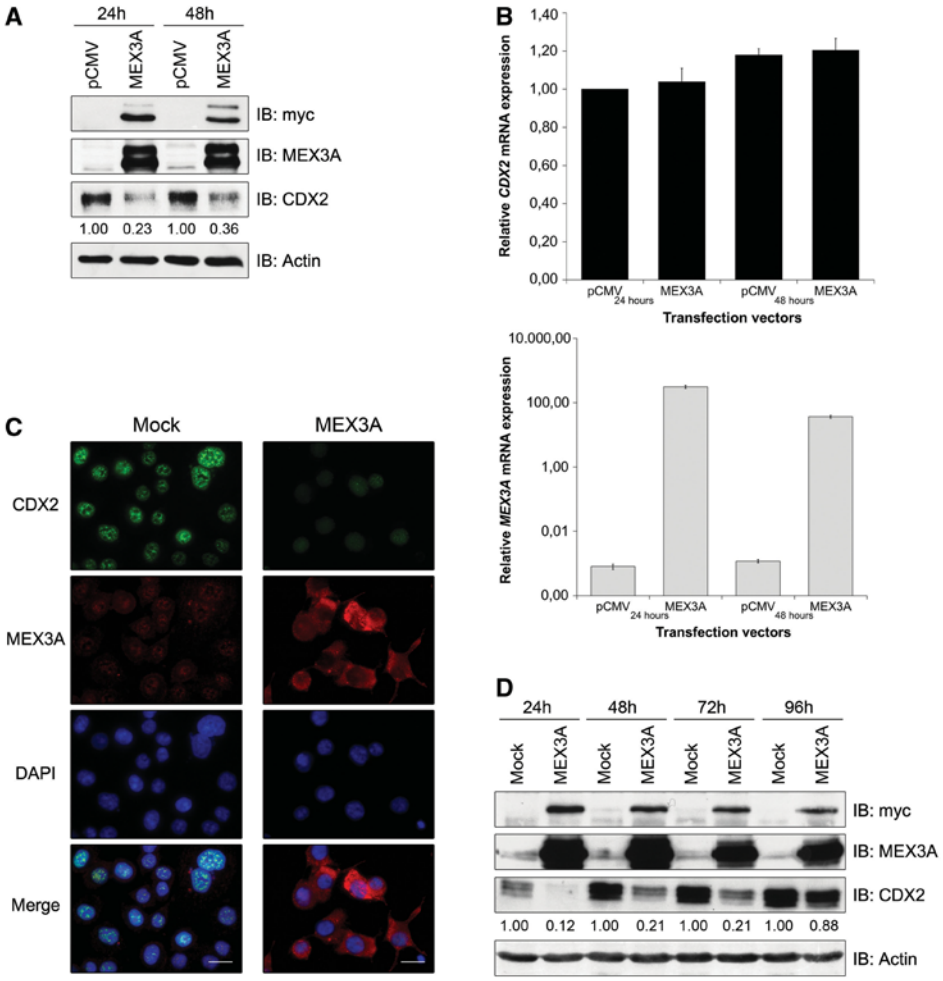

Figure 3. CDX2 regulation by MEX3A in AGS cells. (**A**) Western blot of transient transfections with a myc-tagged MEX3A expression vector. (**B**) qPCR of *CDX2* and *MEX3A* mRNA expression in the previous samples. The value for *CDX2* mRNA expression in the empty vector transfected cells at 24 h was referred to as 1. (**C**) Immunofluorescence for MEX3A and CDX2 in MEX3A stably transfected cells at 48 h culture (original magnification, x400; scale bar 20 µm). (**D**) Western blot of MEX3A and CDX2 expression in stably transfected cells at different time points.

It was previously published that MEX3A and MEX3B were novel components of P bodies (28,29), discrete cytosolic foci characterized in yeast and mammals as centres for translational silencing and mRNA decay (41). In our setting, on MEX3A-induced CDX2 protein decrease, we observed a specific subcellular localization of MEX3A in P bodies, as confocal microscopy analysis revealed a partial overlap between myc-tag staining and two known P body components, the human mRNA-decapping enzyme 1A (DCP1A) and the human enhancer of mRNA-decapping protein 4 (EDC4) (Supplementary Figure S1). Together, these results demonstrate that MEX3A has the ability to regulate CDX2 expression at the post-transcriptional level, probably by interplay with P bodies.

MEX3A interacts with a MRE present in *CDX2* 3`UTR

A bioinformatics search conducted throughout the *CDX2* 3`UTR led to the identification of the MEX-3 degenerate consensus binding sequence (36). The MRE present in *CDX2* mRNA was defined as a bipartite element that consists of AGAG and UUUA motifs separated by two Uracil bases (Figure 4A). To assess whether MEX3A associates with CDX2 3`UTR, we preserved RNA–protein interactions by ultraviolet covalent linkage in AGS cells transiently transfected with the MEX3A expression vector and performed an RNA-immunoprecipitation (RIP) assay. We found a significant enrichment in *CDX2* mRNA recovered with the anti-myc antibody compared with control IgG (Figure 4B). On the contrary, no enrichment for *GAPDH* mRNA was observed (Figure 4B). Likewise, no enrichment for *TBP* mRNA and *18S* rRNA was detected (data not shown). Next, we asked whether MEX3A binds specifically to the MRE present within the *CDX2* 3`UTR. To address this question, *Rluc* expression vectors containing the wild-type *CDX2* 3`UTR (pRLCDX2) or the CDX2 3`UTR with a mutated MRE (pRLΔCDX2) were used in an RIP assay as before (Figure 4C). A complete loss in MEX3A binding

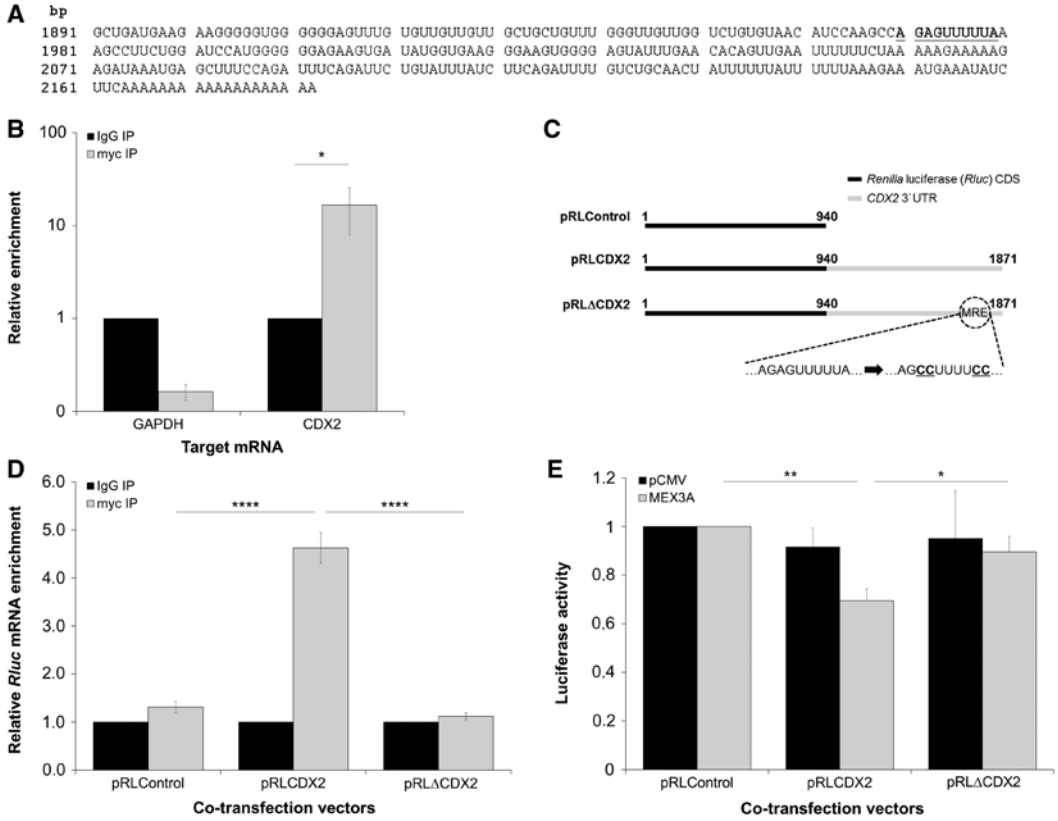

Figure 4. Mechanistics of MEX3A interaction with *CDX2* mRNA in AGS cells. (**A**) Terminal sequence of *CDX2* mRNA, with the predicted MRE in the 3`UTR highlighted in bold and underlined. (**B**) qPCR showing *CDX2* and *GAPDH* mRNAs immunoprecipitated with myc-tag antibody in MEX3A transfected cells (**P* = 0.018). The values for *CDX2* and *GAPDH* mRNA levels in the IgG sample were referred to as 1. (**C**) Schematic representation of pRL constructs. (**D**) qPCR showing *Rluc* mRNA immunoprecipitated with myc-tag antibody for pRLCDX2 transfection (*****P* < 0.0001). Values for *Rluc* mRNA expression in IgG samples were referred to as 1. (**E**) Luciferase activity assay for the different pRL constructs (***P* = 0.006 for pRLControl/pRLCDX2 and **P* = 0.036 for pRLCDX2/pRLΔCDX2). The values obtained for luciferase expression in the pRLControl co-transfected cells were referred to as 1.

capacity was detected when the MRE was mutated, as observed by comparing the levels of *Rluc* mRNA recovered with anti-myc antibody between the pRLCDX2 and pRLΔCDX2 transfected cells (Figure 4D). Additionally, luciferase activity assays proved that the construct with the mutated MRE was insensitive to MEX3A function, as a significant reduction in *Renilla* expression was only detected with the pRLCDX2 transfec- tion (Figure 4E). We conclude that MEX3A is able to interact with *CDX2* mRNA and that the major determinant for this interaction is a putative MRE located in the transcript 3`UTR.

Intestinal phenotype of Caco-2 cells is affected by modulating MEX3A levels

To assess the functional consequences of the previous observations and confirm that they were cell-type independent, we studied CDX2 regulation by MEX3A in another model. Caco-2 cell line was chosen for its ability to spontaneously differentiate into an enterocytic-like phenotype on reaching confluence, which has turned it into the most widely used system to study intestinal cell maturation (42). Furthermore, this process was previously shown to be accompanied by an increase in CDX2 protein expression that, in some established Caco-2 clones, is not explained by higher transcriptional levels (20). First, we evaluated the timing of CDX2 and MEX3A expression in Caco-2, harvesting cells at pre-confluence (day -2), confluence (day 0) and post-confluence (day 2–8) time points. Consistent with previous reports, CDX2 protein expression progressively increased starting from confluence and during differentiation (Supplementary Figure S2A). This trend was also verified at the mRNA level, though not to the same extent (Supplementary Figure S2B). By contrast, *MEX3A* mRNA expression varied in a complementary manner to that of CDX2, with a decreased expression associated to more differentiated cells (Supplementary Figure S2B), although a protein decrease was only visible at day 8 (Supplementary Figure S2A). We inhibited endogenous MEX3A using siRNA and observed that CDX2 protein started to be highly expressed at earlier time points in cells with downregulated MEX3A expression (Figure 5A). Paradoxically, this effect was reverted from confluence onwards, with lower CDX2 protein levels

detected at later time points in cells with MEX3A inhibition (Figure 5A). To evaluate whether this result was dependent on MEX3A activity or due to transcriptional regulation, *CDX2* mRNA expression was assessed. As expected, *CDX2* mRNA levels were not altered in the pre-confluence time points but were downregulated from confluence onwards (Figure 5B), suggesting that after confluence, other MEX3A regulatory functions indirectly interfere with endogenous CDX2 levels.

It is known that Caco-2 cells acquire expression of different intestinal columnar lineage markers on differentiation, such as Villin. To determine the effect of MEX3A overexpression in intestinal differentiation, we generated a Caco-2 cell line stably transfected with the myc-tagged MEX3A construct. Expression of the myc-tag was confirmed in different confluence states, and a concomitant reduction in CDX2 protein levels was observed (Figure 5C). Furthermore, we detected decreased Villin expression in MEX3A overexpressing cells (Figure 5C).

As differentiation and growth arrest are spatially and temporally related events in the intestinal epithelium, we studied the expression of cyclin D1, a positive regulator of proliferation required for cell cycle G1/S transition. Sustained levels of cyclin D1 were detected in MEX3A overexpressing cells in latter time points, when the culture is normally already less proliferative, as observed by the striking decrease of cyclin D1 in mock cells (Figure 5C). To ascertain the cell cycle distribution related to MEX3A overexpression, we transiently transfected near-confluent Caco-2 cells with the myc-tagged MEX3A construct and performed DNA content analysis by flow cytometry. Measurements indicated that cells overexpressing MEX3A possess a different cell cycle profile (Figure 5D), with a reduced G0/G1 population (32 against 49% in the empty transfected cells) and an increased S phase population (41 against 30% in the empty transfected cells). Given the observed alterations in differentiation and proliferation parameters, we

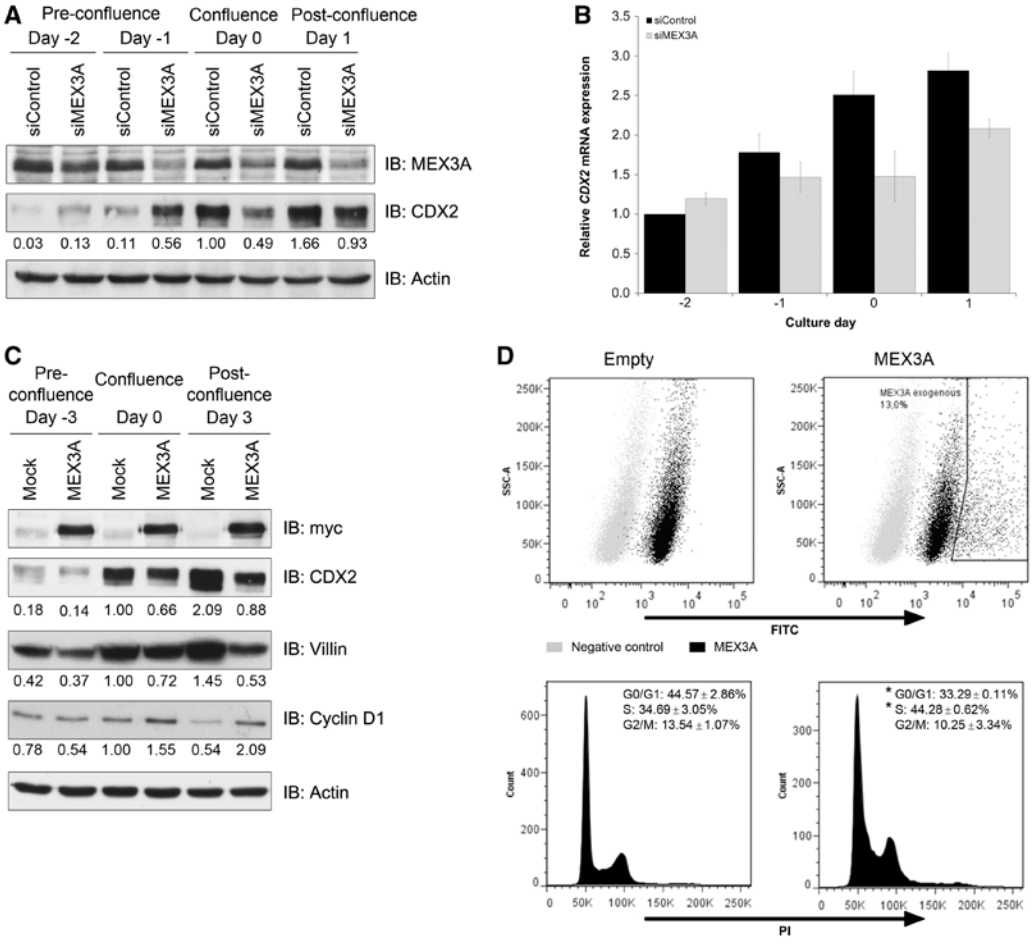

Figure 5. MEX3A modulation of intestinal phenotype in Caco-2 cells. (**A**) Western blot of MEX3A inhibition with transfection of siRNAs performed at day -3 and day -1. (**B**) qPCR of *CDX2* mRNA expression in the same samples. The value for *CDX2* at day -2 for the siControl sample was referred to as 1. (**C**) Western blot of MEX3A stably transfected cells at different confluences for differentiation and proliferation markers. (**D**) Flow cytometry analysis of MEX3A transiently transfected cells. Dot plots depicting negative control (secondary antibody only) and FITC-conjugated MEX3A expression levels are shown on top. DNA content histograms showing population percentages for the different cell cycle phases of the empty vector transfected cells and MEX3A-transfected cells (gate MEX3A exogenous) are shown below (*P = 0.03 for G0/G1 phase and *P = 0.05 for S phase).

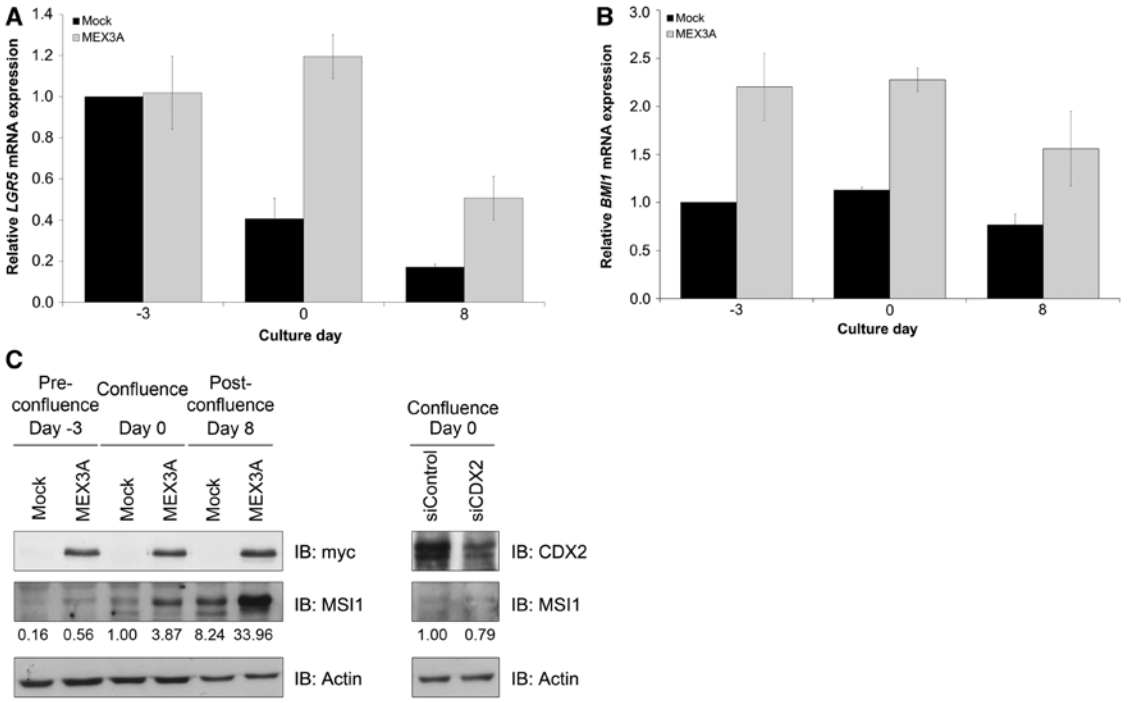

Figure 6. Expression profiles of intestinal stem cell markers in Caco-2 cells. (**A**) qPCR of *LGR5* mRNA expression in mock and MEX3A overexpressing cells. The value for *LGR5* at day -3 for the mock sample was referred to as 1. (**B**) qPCR of *BMI1* mRNA expression in the same samples. The value for *BMI1* at day -3 for the mock sample was referred to as 1. (**C**) Western blot analysis of MSI1 expression in the same samples and in Caco-2 parental cell line with CDX2 inhibition by siRNAs at the confluence time point.

sought to determine whether MEX3A overexpressing cells possessed intestinal 'progenitor or stem-like' features. Assessment of expression of well-established intestinal stem cell markers (43–46) revealed that these cells show overall increased levels of *LGR5*, *BMI1* and MSI1 (Figure 6), whereas *OLFM4* basal expression was barely detectable and did not change (data not shown). Importantly, this seems to be independent of CDX2, as siRNA-mediated CDX2 downregulation did not reproduce the increase in MSI1 levels (Figure 6C).

Caco-2 cells retain the ability to polarize and form a transporting epithelial monolayer in culture (42). This polarization process is intimately connected with the formation of cell–cell contacts and adhesion. In standard culture conditions, Caco-2 cells overexpressing MEX3A showed alterations in the distribution of the tight junction marker ZO-1 compared with the mock cells (Figure 7A). We then used a 3D cell cyst assay (4) in matrigel to compare the phenotypic response of both cell lines. We observed that mock cells developed cyst-like structures in a few days, with ~50% showing a well-defined central lumen (Figure 7B–D). In contrast, >80% of the MEX3A-expressing cysts, in which CDX2 reduction and MEX3A increase was confirmed, failed to elaborate such a lumen (Figure 7B–D). Additionally, we observed distinct patterns of E-cadherin and Phalloidin expression. We detected basolateral E-cadherin expression and accumulation of filamentous-actin (F-actin) around the hollow lumen in the mock cysts (Figure 7D), and a reduced E-cadherin expression in the MEX3A-expressing cysts, with F-actin appearing ubiquitously distributed (Figure 7D). Thus, we show that the establishment of proper apical-basal identity is affected on MEX3A overexpression.

MEX3A is differentially expressed in the crypt-villus unit

To address MEX3A relevance *in vivo*, we analysed its presence in mouse normal intestine and compared it with CDX2 expression. We observed that MEX3A is expressed in small intestine and colon, exhibiting mainly a nuclear staining pattern. It shows a stronger expression in the lower portion of the crypt-villus unit in small intestine and reduced expression in the uppermost part of the colonic crypts (Figure 8). In the small intestine, CDX2 is expressed in the same cells as MEX3A except in the base of the crypts (Figure 8, insert) that exhibit mainly MEX3A protein. In colon, CDX2 is more expressed in the surface cells (Figure 8). These results suggest that MEX3A expression might be important to define intestinal differentiation patterns.

DISCUSSION

In this study, we provide the first demonstration that MEX3A represses CDX2 expression in the gastrointestinal context, putatively as a translational repressor, with direct implications in intestinal differentiation, polarity and stemness features, key mechanisms for tissue homeostasis that are frequently altered in pathological conditions and inextricably linked with cancer.

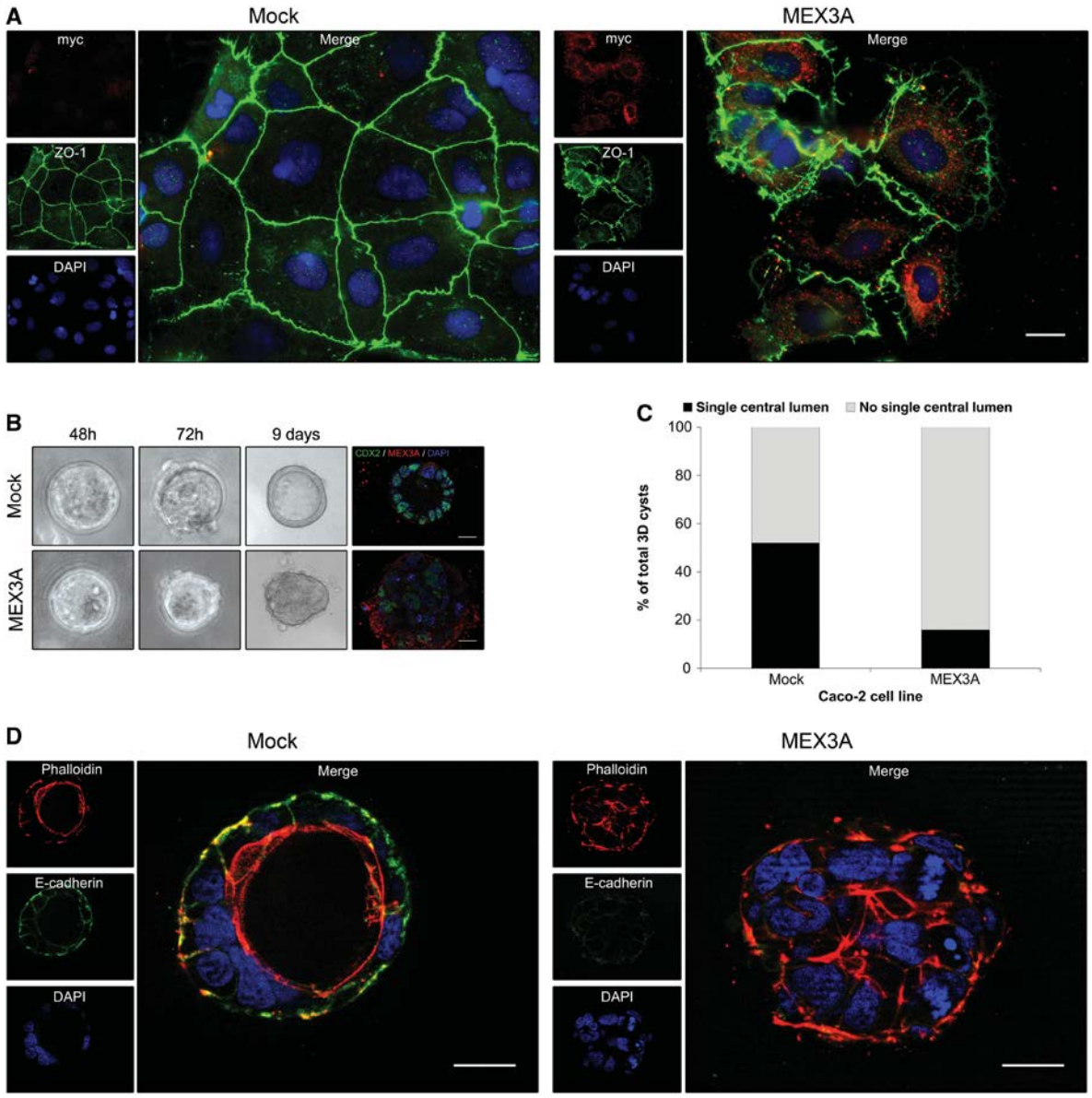

Figure 7. Polarity alterations induced by MEX3A in Caco-2 cells. (**A**) Immunofluorescence showing ZO-1 expression in Caco-2 mock and MEX3A stably transfected cell lines at day -2 of culture (original magnification, x400). (**B**) Morphology of Caco-2 mock and MEX3A cysts in bright field microscopy during 3D culture (original magnification, x100) and CDX2/MEX3A expression (original magnification, x630). (**C**) Quantification of cysts with lumen or no lumen at culture day 8. (**D**) Expression of E-cadherin and Phalloidin staining in Caco-2 cysts (original magnification, x630; all scale bars 20 µm).

3D cell culture systems have been shown to enable physiological and functional differentiation of several epithelial cell types (47,48), constituting a promising alternative to overcome standard cell culture limitations. Accordingly, our transcriptomic analysis performed on a 3D model of AGS cells, which have a significant level of endogenous CDX2 responsive to different molecular stimuli (13–15), allowed us to disclose MEX3A as a molecular player involved in the regulation of CDX2 translation. The array presented an increased MEX3A expression in 3D culture and no alteration in the levels of the other MEX3 family members. We further demonstrated that MEX3A overexpression leads to marked CDX2 protein decrease in two cell lines. We tried to overcome the limitation of using overexpression systems by using two different cell lines that were both transiently and stably transfected with a MEX3A plasmid, which gave concordant results. In addition, CDX2 negative regulation by MEX3A was confirmed in a more physiological context using a siRNA approach towards endogenous MEX3A in Caco-2 cells. We proved that MEX3A is able to interact with *CDX2* mRNA through a canonical MRE present

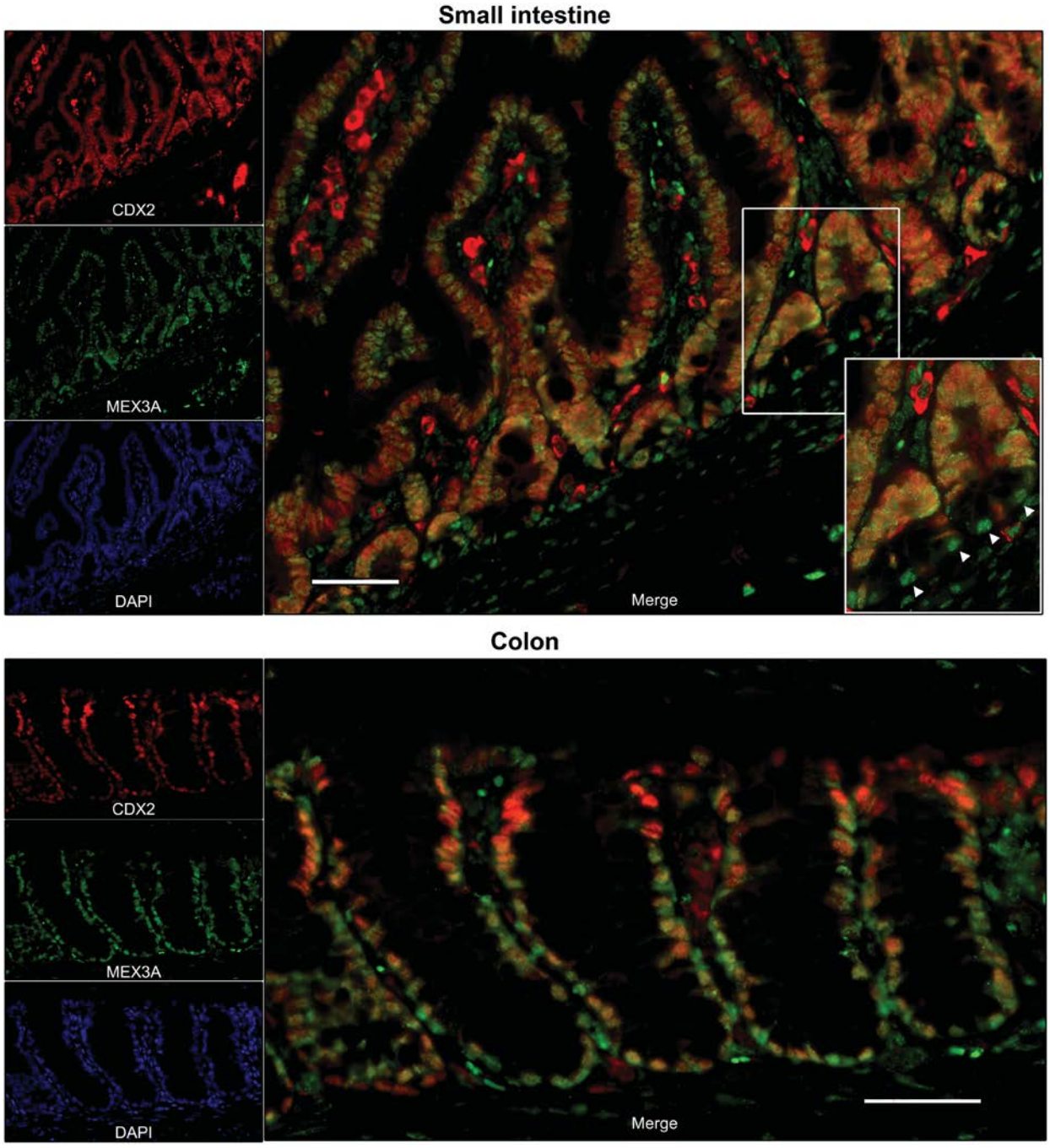

Figure 8. Expression patterns of MEX3A and CDX2 in mouse normal intestine. Representative immunofluorescence data for MEX3A and CDX2 in small intestine and colon are shown (original magnification, x200; scale bars 50 μm; insert original magnification, x400).

in the 3´UTR. This sequence, which we now show to be functionally relevant in humans, seems to be the single determinant of MEX3A binding, independently of the upstream 5`coding region. In fact, bioinformatics analysis shows that the degenerate MRE motif has been evolutionarily conserved in different CDX2 homologues (Supplementary Figure S3), namely in chimpanzee, mouse, rat, zebrafish, fruit fly and frog, suggesting that MEX3A is critical for CDX2 regulation.

Endogenously, MEX3A was mainly localized in the nuclear compartment of intestinal epithelial cells, both *in vitro* and *in vivo*. This result is crucial to show that a biological background for the regulation of CDX2 by MEX3A exists. In this regard, a variant form of MEX3D

called TINO, which has been shown to negatively regulate BCL-2 expression by transcript destabilization, is also predominantly localized in the nuclei of HeLa cells (33). On the other hand, MEX3A transfectants showed predominant cytoplasmic staining. A potential problem could be antibody specificity; however, this was successfully evaluated by transfection of siRNA duplexes directed against MEX3A (Supplementary Figure S4). Therefore, the differential localization in distinct expression backgrounds might be because a certain threshold in expression levels has to be achieved for cytoplasmic translocation to occur, given that MEX3 proteins are capable of performing nucleocytoplasmic shuttling (28). On the other hand, specific subcellular distribution might be related with protein phosphorylation, in accordance with published data describing MEX3 members as phosphoproteins (28,29). Whichever the case, this does not contradict post-transcriptional regulation, as it can be elicited at multiple points of the transcript lifespan, including pre-mRNA processing in the nucleus, export from the nucleus to the cytoplasm and subsequent coordinated trafficking of the mature mRNA to the translation machinery. It also remains to be fully clarified the consequence of partial accumulation in P bodies, structures involved in processes of mRNA degradation, nonsense-mediated mRNA decay, translational repression and RNA-mediated gene silencing (49), although there are several RBPs with established roles in translational regulation known to colocalize with P bodies (50,51).

We used the Caco-2 cell line model to modulate MEX3A expression and assess its phenotype. Surprisingly, MEX3A inhibition produced distinct effects over CDX2 levels depending on cellular confluence. MEX3A might selectively regulate unique subsets of targets in different culture conditions. In agreement, it is known that transcriptomic and proteomic changes occur during the progression from the proliferative state to spontaneous differentiation of Caco-2 cells. Conversely, MEX3A might have a dual role, acting both as a repressor or enhancer contingent on the cellular microenvironment, as observed for other RBPs, like HuR (52). It is possible that intricate associations of MEX3A with other molecular effectors, namely other proteins or microRNAs, determine divergent regulatory endings. MEX3A overexpression in Caco-2 cells resulted in pronounced phenotypic alterations. CDX2 and Villin downregulation were indicative of a loss in intestinal differentiation, which was further confirmed by the flow cytometric profile, showing reduced G0/G1 population, usually associated with less-differentiated cells. Another hallmark feature of MEX3A overexpression was the altered cellular polarization in standard culture, as well as the impaired ability to form polarized structures in the presence of matrigel. These effects may be mediated by CDX2, as this transcription factor was previously shown to regulate intestinal Villin through recruitment of the Brm-type SWI/SNF complex to its promoter (53), and MEX3A-expressing cysts closely resemble the ones obtained with Caco-2 cells in which CDX2 suppression was achieved by lentiviral short-hairpin RNA particles (4). It is, therefore, important to ascertain the biological setting where this regulation might be determinant, which is suggested by the predominant expression of MEX3A in the stem, transit-amplifying and migrating post-mitotic cells of the intestine. Although CDX2 protein can be detected in most of these cells, its level is lower in the crypts compared with the uppermost differentiated cells of the villi (2,19,20). A similar increasing bottom-up gradient has been described along the colonic gland axis in the distal colon epithelium. By contrast, *in situ* hybridization revealed that *CDX2* mRNA was homogeneously distributed along the entire crypt-villus axis (20). Given this CDX2 protein expression gradient, that we also show, and lack of correlation with mRNA, it is likely that MEX3A fine-tunes CDX2 levels in vivo as well, in a transcription-independent manner, providing swift availability of the protein to meet the physiological requirements of the continuously renewed gut epithelium. Moreover, it has been shown that intestinal stem cells cannot differentiate into any of the intestinal lineages in a background of Cdx2 ablation (54), revealing the need for tight CDX2 regulation as determinant to proper phenotype switching. Most interestingly, we observed higher expression of different intestinal stem cell markers when we overexpressed MEX3A in Caco-2, suggesting that this protein is associated with stem cell features. Our AGS 3D model also showed a significant upregulation of *OLFM4* (Supplementary Table S2), previously identified as a marker for LGR5+ stem cells in human intestine (46), although this gene was not directly upregulated in Caco-2 cells, which might be due to intrinsic properties of each cell line. Strengthening the hypothesis for a role of MEX3A in stem cell potential, a recent publication showed that MEX3A is part of the molecular signature of the LGR5+ intestinal stem cells, presenting a 1.64-fold increase in relation to daughter cells, along with MSI1, LGR5 and OLFM4 (55). Furthermore, our study reinforces the increasing knowledge that other regulatory mechanisms in addition to transcriptional ones have important functions in stem cells. Although we do not know yet how MEX3A relates with the intestinal stem cell phenotype, we hypothesize that MEX3A overexpression in itself, together with the induction of a lessened differentiated phenotype and polarity defects mediated by CDX2 downregulation, might be critical to allow a permissive environment for the appearance of stemness features. Furthermore, the MEX3A–CDX2 axis might be important during early embryogenesis where CDX2 is required for correct trophectoderm differentiation, while absent from the inner cell mass, in a process that requires tight regulation (56). Interestingly, the pattern of expression of the *C. elegans* orthologues of both proteins was found to be mutually exclusive in early stages of embryogenesis (24,27). Still, it remains to be assessed the relevance of MEX3A in multiple pathological contexts of the gastrointestinal tract where differentiation abnormalities directed by CDX2 are key events.

In conclusion, we have identified a novel role for MEX3A protein in the regulation of intestinal differentiation, polarity and stemness features, partially mediated by the repression of CDX2. This is the first description of a CDX2 regulatory mechanism based on its mRNA control by an RBP, having a significant impact in

intestinal homeostasis and likely in gastrointestinal carcinogenesis.

ACCESSION NUMBERS

The microarray data from this publication have been submitted to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) and assigned the identifier E-MTAB-1234.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–4.

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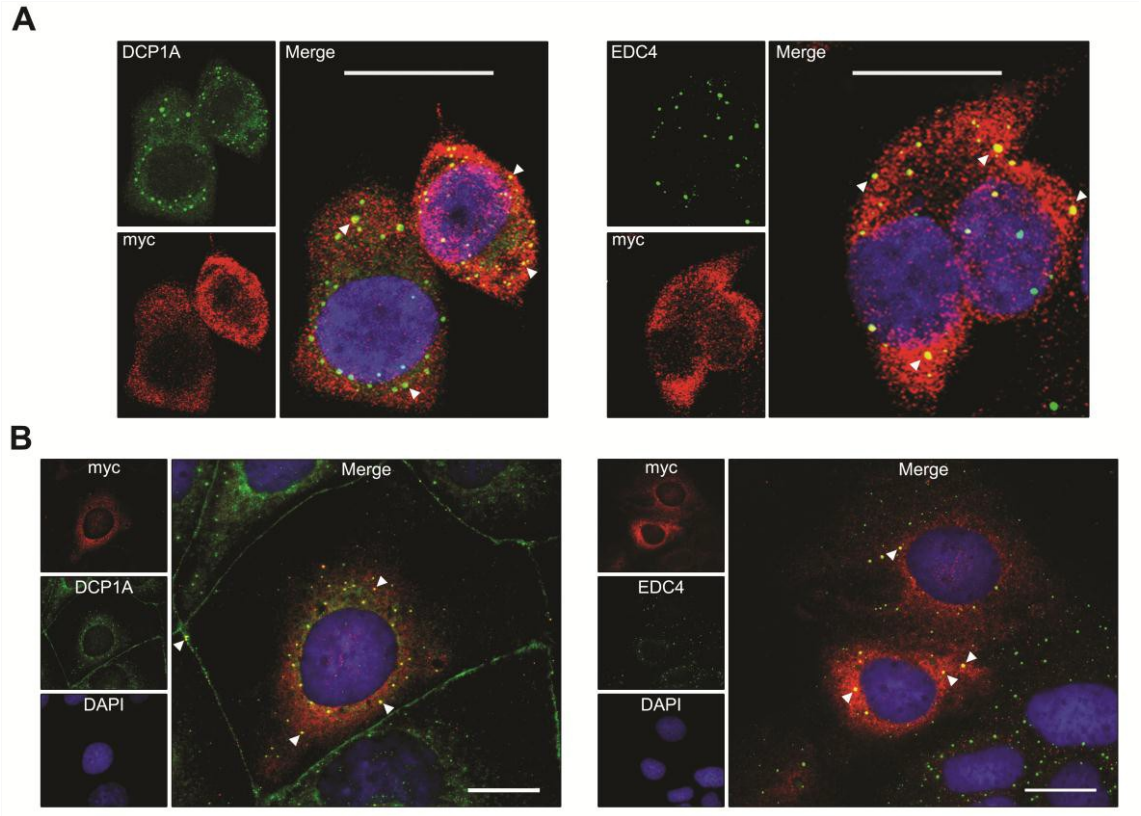
Conflict of interest statement. None declared.

REFERENCES

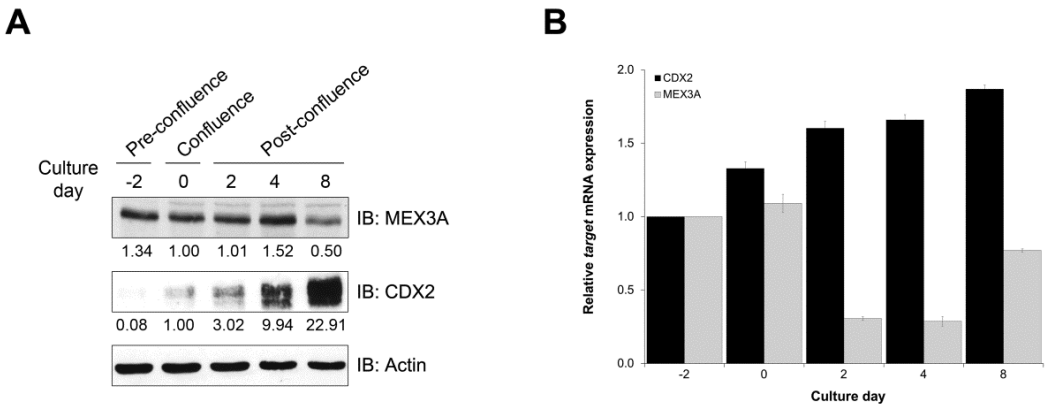
1. Beck,F., Chawengsaksophak,K., Waring,P., Playford,R.J. and Furness,J.B. (1999) Reprogramming of intestinal differentiation and intercalary regeneration in Cdx2 mutant mice. *Proc. Natl Acad. Sci. USA*, **96**, 7318–7323.
2. Silberg,D.G., Swain,G.P., Suh,E.R. and Traber,P.G. (2000) Cdx1 and cdx2 expression during intestinal development. *Gastroenterology*, **119**, 961–971.
3. Gao,N., White,P. and Kaestner,K.H. (2009) Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. *Dev. Cell*, **16**, 588–599.
4. Gao,N. and Kaestner,K.H. (2010) Cdx2 regulates endo-lysosomal function and epithelial cell polarity. *Genes Dev.*, **24**, 1295–1305.
5. Gross,I., Duluc,I., Benameur,T., Calon,A., Martin,E., Brabletz,T., Kedinger,M., Domon-Dell,C. and Freund,J.N. (2008) The intestine-specific homeobox gene Cdx2 decreases mobility and antagonizes dissemination of colon cancer cells. *Oncogene*, **27**, 107–115.
6. Brabletz,T., Spaderna,S., Kolb,J., Hlubek,F., Faller,G., Bruns,C.J., Jung,A., Nentwich,J., Duluc,I., Domon-Dell,C. *et al.* (2004) Down-regulation of the homeodomain factor Cdx2 in colorectal cancer by collagen type I: an active role for the tumor environment in malignant tumor progression. *Cancer Res.*, **64**, 6973–6977.
7. Bonhomme,C., Duluc,I., Martin,E., Chawengsaksophak,K., Chenard,M.P., Kedinger,M., Beck,F., Freund,J.N. and Domon-Dell,C. (2003) The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. *Gut*, **52**, 1465–1471.
8. Eda,A., Osawa,H., Satoh,K., Yanaka,I., Kihira,K., Ishino,Y., Mutoh,H. and Sugano,K. (2003) Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *J. Gastroenterol.*, **38**, 14–22.
9. Almeida,R., Silva,E., Santos-Silva,F., Silberg,D.G., Wang,J., De Bolós,C. and David,L. (2003) Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. *J. Pathol.*, **199**, 36–40.
10. Barros,R., Camilo,V., Pereira,B., Freund,J.N., David,L. and Almeida,R. (2010) Pathophysiology of intestinal metaplasia of the stomach: emphasis on CDX2 regulation. *Biochem. Soc. Trans.*, **38**, 358–363.
11. Silberg,D.G., Sullivan,J., Kang,E., Swain,G.P., Moffett,J., Sund,N.J., Sackett,S.D. and Kaestner,K.H. (2002) Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. *Gastroenterology*, **122**, 689–696.
12. Mutoh,H., Hakamata,Y., Sato,K., Eda,A., Yanaka,I., Honda,S., Osawa,H., Kaneko,Y. and Sugano,K. (2002) Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice. *Biochem. Biophys. Res. Commun.*, **294**, 470–479.
13. Barros,R., Pereira,B., Duluc,I., Azevedo,M., Mendes,N., Camilo,V., Jacobs,R.J., Paulo,P., Santos-Silva,F., van Seuningen,I. *et al.* (2008) Key elements of the BMP/SMAD pathway co-localize with CDX2 in intestinal metaplasia and regulate CDX2 expression in human gastric cell lines. *J. Pathol.*, **215**, 411–420.
14. Camilo,V., Barros,R., Sousa,S., Magalhães,A.M., Lopes,T., Santos,A.M., Pereira,T., Figueiredo,C., David,L. and Almeida,R. (2012) Helicobacter pylori and the BMP pathway regulate CDX2 and SOX2 expression in gastric cells. *Carcinogenesis*, **10**, 1985–1992.
15. Barros,R., da Costa,L.T., Pinto-de-Sousa,J., Duluc,I., Freund,J.N., David,L. and Almeida,R. (2011) CDX2 autoregulation in human intestinal metaplasia of the stomach: impact on the stability of the phenotype. *Gut.*, **60**, 290–298.
16. Benahmed,F., Gross,I., Gaunt,S.J., Beck,F., Jehan,F., Domon-Dell,C., Martin,E., Kedinger,M., Freund,J.N. and Duluc,I. (2008) Multiple regulatory regions control the complex expression pattern of the mouse Cdx2 homeobox gene. *Gastroenterology*, **135**, 1238–1247.
17. Woodford-Richens,K.L., Halford,S., Rowan,A., Bevan,S., Aaltonen,L.A., Wasan,H., Bicknell,D., Bodmer,W.F., Houlston,R.S. and Tomlinson,I.P. (2001) CDX2 mutations do not account for juvenile polyposis or Peutz-Jeghers syndrome and occur infrequently in sporadic colorectal cancers. *Br. J. Cancer*, **84**, 1314–1316.
18. Pereira,B., Oliveira,C., David,L. and Almeida,R. (2009) CDX2 promoter methylation is not associated with mRNA expression. *Int. J. Cancer*, **125**, 1739–1742.
19. Rings,E.H., Boudreau,F., Taylor,J.K., Moffett,J., Suh,E.R. and Traber,P.G. (2001) Phosphorylation of the serine 60 residue within the Cdx2 activation domain mediates its transactivation capacity. *Gastroenterology*, **121**, 1437–1450.
20. Boulanger,J., Vézina,A., Mongrain,S., Boudreau,F., Perreault,N., Auclair,B.A., Lainé ,J., Asselin,C. and Rivard,N. (2005) Cdk2-dependent phosphorylation of homeobox transcription

factor CDX2 regulates its nuclear translocation and proteasome-mediated degradation in human intestinal epithelial cells. *J. Biol. Chem.*, **280**, 18095–18107.
21. Moore,M.J. (2005) From birth to death: the complex lives of eukaryotic mRNAs. *Science*, **309**, 1514–1518.
22. Besse,F. and Ephrussi,A. (2008) Translational control of localized mRNAs: restricting protein synthesis in space and time. *Nat. Rev. Mol. Cell. Biol.*, **9**, 971–980.
23. Dreyfuss,G., Kim,V.N. and Kataoka,N. (2002) Messenger-RNA-binding proteins and the messages they carry. *Nat. Rev. Mol. Cell. Biol.*, **3**, 195–205.
24. Draper,B.W., Mello,C.C., Bowerman,B., Hardin,J. and Priess,J.R.(1996) MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. *Cell*, **87**, 205–216.
25. Ciosk,R., DePalma,M. and Priess,J.R. (2006) Translational regulators maintain totipotency in the Caenorhabditis elegans germline. *Science*, **311**, 851–853.
26. Siomi,H., Matunis,M.J., Michael,W.M. and Dreyfuss,G. (1993) The pre-mRNA binding K protein contains a novel evolutionary conserved motif. *Nucleic Acids Res.*, **21**, 1193–1198.
27. Hunter,C.P. and Kenyon,C. (1996) Spatial and temporal controls target pal-1 blastomere-specification activity to a single blastomere lineage in C. elegans embryos. *Cell*, **87**, 217–226.
28. Buchet-Poyau,K., Courchet,J., Le Hir,H., Séraphin,B., Scoazec,J.Y., Duret,L., Domon-Dell,C., Freund,J.N. and Billaud,M. (2007) Identification and characterization of human Mex-3 proteins, a novel family of evolutionarily conserved RNA-binding proteins differentially localized to processing bodies. *Nucleic Acids Res.*, **35**, 1289–1300.
29. Courchet,J., Buchet-Poyau,K., Potemski,A., Brès,A., Jariel-Encontre,I. and Billaud,M. (2008) Interaction with 14-3-3 adaptors regulates the sorting of hMex-3B RNA-binding protein to distinct classes of RNA granules. *J. Biol. Chem.*, **283**, 32131–32142.
30. Cano,F., Bye,H., Duncan,L.M., Buchet-Poyau,K., Billaud,M., Wills,M.R. and Lehner,P.J. (2012) The RNA-binding E3 ubiquitin ligase MEX-3C links ubiquitination with MHC-I mRNA degradation. *EMBO J.*, **31**, 3596–3606.
31. Jiao,Y., Bishop,C.E. and Lu,B. (2012) Mex3c regulates insulin-like growth factor 1 (IGF1) expression and promotes postnatal growth. *Mol. Biol. Cell*, **23**, 1404–1413.
32. Jiao,Y., George,S.K., Zhao,Q., Hulver,M.W., Hutson,S.M., Bishop,C.E. and Lu,B. (2012) Mex3c mutation reduces adiposity and increases energy expenditure. *Mol. Cell. Biol.*, **32**, 4350–4362.
33. Donnini,M., Lapucci,A., Papucci,L., Witort,E., Jacquier,A., Brewer,G., Nicolin,A., Capaccioli,S. and Schiavone,N. (2004) Identification of TINO: a new evolutionary conserved BCL-2 AU-rich element RNA-binding protein. *J. Biol. Chem.*, **279**, 20154–20166.
34. Jiang,H., Zhang,X., Luo,J., Dong,C., Xue,J., Wei,W., Chen,J., Zhou,J., Gao,Y. and Yang,C. (2012) Knockdown of hMex-3A by small RNA interference suppresses cell proliferation and migration in human gastric cancer cells. *Mol. Med. Report.*, **6**, 575–580.
35. Pillai,R.S., Bhattacharyya,S.N., Artus,C.G., Zoller,T., Cougot,N.,Basyuk,E., Bertrand,E. and Filipowicz,W. (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*, **309**, 1573–1576.
36. Pagano,J.M., Farley,B.M., Essien,K.I. and Ryder,S.P. (2009) RNA recognition by the embryonic cell fate determinant and germline totipotency factor MEX-3. *Proc. Natl Acad. Sci. USA*, **106**, 20252–20257.
37. Simon,R., Lam,A., Li,M.C., Ngan,M., Menenzes,S. and Zhao,Y. (2007) Analysis of gene expression data using BRB-array tools. *Cancer Inform.*, **3**, 11–17.
38. Saeed,A.I., Sharov,V., White,J., Li,J., Liang,W., Bhagabati,N., Braisted,J., Klapa,M., Currier,T., Thiagarajan,M. *et al.* (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques*, **34**, 374–378.
39. Huang,D.W., Sherman,B.T. and Lempicki,R.A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.*, **4**, 44–57.
40. Schindelin,J., Arganda-Carreras,I., Frise,E., Kaynig,V., Longair,M., Pietzsch,T., Preibisch,S., Rueden,C., Saalfeld,S., Schmid,B. *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods*, **9**, 676–682.
41. Sheth,U. and Parker,R. (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science*, **300**, 805–808.
42. Pinto,M., Robine-Lé on,S., Appay,M.D., Kedinger,M., Triadou,N., Dussaulx,E., Lacroix,B., Simon-Assmann,P., Haffen,K., Fogh,J. *et al.* (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell*, **47**, 323–330.
43. Potten,C.S., Booth,C., Tudor,G.L., Booth,D., Brady,G., Hurley,P., Ashton,G., Clarke,R., Sakakibara,S. and Okano,H. (2003) Identification of a putative intestinal stem cell and early lineage marker; musashi-1. *Differentiation*, **71**, 28–41.
44. Barker,N., van Es,J.H., Kuipers,J., Kujala,P., van den Born,M., Cozijnsen,M., Haegebarth,A., Korving,J., Begthel,H., Peters,P.J. *et al.* (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*, **449**, 1003–1007.
45. Sangiorgi,E. and Capecchi,M.R. (2008) Bmi1 is expressed in vivo in intestinal stem cells. *Nat. Genet.*, **40**, 915–920.
46. van der Flier,L.G., Haegebarth,A., Stange,D.E., van de Wetering,M. and Clevers,H. (2009) OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology*, **137**, 15–17.
47. Bissell,M.J., Radisky,D.C., Rizki,A., Weaver,V.M. and Petersen,O.W. (2002) The organizing principle: microenvironmental influences in the normal and malignant breast. *Differentiation*, **70**, 537–546.
48. Ootani,A., Toda,S., Fujimoto,K. and Sugihara,H. (2003) Foveolar differentiation of mouse gastric mucosa in vitro. *Am. J. Pathol.*, **162**, 1905–1912.
49. Eulalio,A., Behm-Ansmant,I. and Izaurralde,E. (2007) P bodies: at the cross-roads of post-transcriptional pathways. *Nat. Rev. Mol. Cell. Biol.*, **8**, 9–22.
50. Chu,C.Y. and Rana,T.M. (2006) Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS Biol.*, **4**, e210.
51. Yang,W.H., Yu,J.H., Gulick,T., Bloch,K.D. and Bloch,D.B. (2006) RNA-associated protein 55 (RAP55) localizes to mRNA processing bodies and stress granules. *RNA*, **12**, 547–554.
52. Kim,H.H., Kuwano,Y., Srikantan,S., Lee,E.K., Martindale,J.L. and Gorospe,M. (2009) HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev.*, **23**, 1743–1748.
53. Yamamichi,N., Inada,K., Furukawa,C., Sakurai,K., Tando,T., Ishizaka,A., Haraguchi,T., Mizutani,T., Fujishiro,M., Shimomura,R. *et al.* (2009) Cdx2 and the Brm-type SWI/SNF complex cooperatively regulate villin expression in gastrointestinal cells. *Exp. Cell Res.*, **315**, 1779–1789.
54. Stringer,E.J., Duluc,I., Saandi,T., Davidson,I., Bialecka,M., Sato,T., Barker,N., Clevers,H., Pritchard,C.A., Winton,D.J. *et al.* (2012) Cdx2 determines the fate of postnatal intestinal endoderm. *Development*, **139**, 465–474.
55. Muñoz,J., Stange,D.E., Schepers,A.G., van de Wetering,M., Koo,B.K., Itzkovitz,S., Volckmann,R., Kung,K.S., Koster,J., Radulescu,S. *et al.* (2012) The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. *EMBO J.*, **31**, 3079–3091.
56. Strumpf,D., Mao,C.A., Yamanaka,Y., Ralston,A., Chawengsaksophak,K., Beck,F. and Rossant,J. (2005) Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development*, **132**, 2093–2102.

SUPPLEMENTARY DATA



Supplementary Figure S1. Subcellular localization of MEX3A in P bodies (**A**) Immunofluorescence of MEX3A stably transfected AGS cells for myc-tag and for endogenous DCP1A and EDC4 proteins. (**B**) Immunofluorescence of MEX3A stably transfected Caco-2 cells for myc-tag and endogenous DCP1A and EDC4 proteins. White arrow-heads point to different sites of co-localization (original magnification, x630; scale bars 20 μm).



Supplementary Figure S2. Study of the expression profile of endogenous MEX3A in Caco-2 cells. (**A**) Western blot of MEX3A and CDX2 expression during Caco-2 differentiation. The time-point in which confluence is achieved is referred to as day 0 (culture day 6). (**B**) qPCR of *MEX3A* and *CDX2* mRNA expression during the same time-points. Values for *CDX2* and *MEX3A* at day -2 were referred to as 1.

Pan troglodytes CDX2 - One predicted MRE in the 3`UTR

bp	Sequence
1991	AGAGTTTTTA

Mus musculus Cdx2 - Two predicted MREs in the CDS

bp	Sequence
873	GGAGTTTCACTTTA
1171	GGAGGGGTTTTA

Rattus norvegicus Cdx2 - Three predicted MREs, one in the CDS and two in the 3`UTR

bp	Sequence
1010	GGAGGGGTTTTA
1228	TTAGATTTTTTTTTTA
2027	GGAGCTTTA

Danio rerio cdx1a - One predicted MRE in the 3`UTR

bp	Sequence
1176	TGAGTTTA

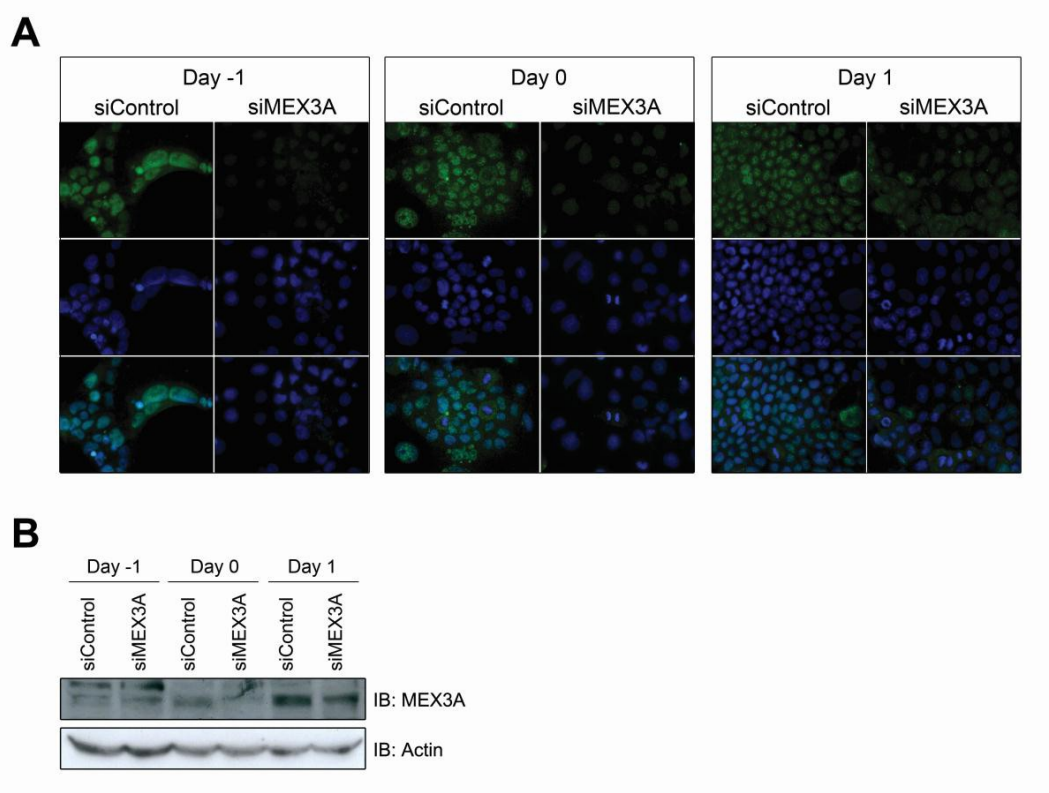
Xenopus laevis cdx2 - Seven predicted MREs in the 3`UTR

bp	Sequence
1269	ATAGACTTTTA
1640	TTAGATCCCTTCTA
1860	GTAGCATTTTA
2251	GTAGGCAGCCTTTA
2355	GGAGACGTTTA
2488	TGAGTGTTTTATA
2546	GGAGGATATATTTA

Drosophila melanogaster cad - Four predicted MREs in the 3´UTR

bp	Sequence
2235	ATAGCCGCATATATA
2251	AGAGTTTTAACGTTTA
2298	GTAGTTAATATA
2469	ATAGCTATTTA

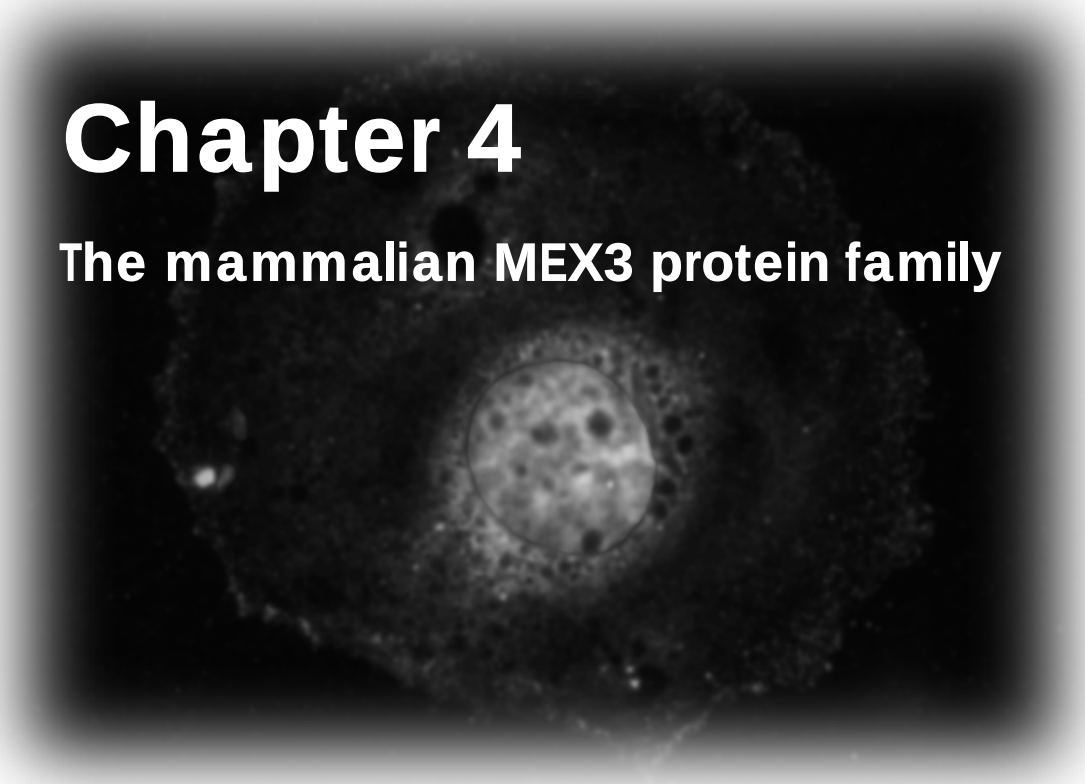
Supplementary Figure S3. Bioinformatics search for MREs in *CDX2* homologues of several species. Different MRE sequences located in the coding sequence (CDS) or 3`UTR of the transcripts are shown.



Supplementary Figure S4. Inhibition of endogenous MEX3A in Caco-2 cells. (**A**) Immunofluorescence for endogenous MEX3A at different time-points, upon inhibition with specific siRNAs for 24h (original magnification, x400; scale bar 20 µm). (**B**) Corresponding western blot analysis of MEX3A downregulation.

Chapter 4

The mammalian MEX3 protein family



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Spotlight

Cell PRESS

MEX-3 proteins: recent insights on novel post-transcriptional regulators

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RNA-binding proteins of the evolutionarily-conserved MEX-3 family are mediators of post-transcriptional regulation in different organisms. Recent studies highlight their involvement in diverse physiological settings, including the maintenance of a balance between stem cell self-renewal and differentiation. Here, we draw attention to their putative role in tissue homeostasis and disease, particularly cancer.

The RNA-binding protein MEX-3 is a translational repressor first identified in the nematode *Caenorhabditis elegans*. Vertebrates have four distinct MEX-3 orthologs, and work is ongoing to understand their roles. Among the diverse functions identified thus far, a putative role in self-renewal/differentiation decisions is emerging as a common theme within the human MEX3 family, with implications for stem cell and cancer biology.

C. elegans MEX-3 contributes both to blastomere identity during early embryogenesis and to germline totipotency in adults. Mutations disrupting the *mex-3* locus result in embryos that aberrantly generate body-wall muscles from the anterior blastomere (AB), hence the name *mex* for muscle excess. Normally, the AB contributes to the nervous system, hypodermis, and anterior part of the pharynx, whereas almost all muscle cells derive from the posterior blastomere. Moreover, double mutant strains for *mex-3* and *gld-1*, which encodes a RNA-binding protein required for meiotic progression in germ cells, develop germline tumors containing cells of muscular, neuronal and intestinal nature, reminiscent of human teratomas [1]. These phenotypes are due, in part, to loss of the translational repression that MEX-3 exerts on the mRNA of the transcription factor *pal-1* through interaction with MEX-3 recognition elements (MREs) located in the 3′ untranslated region (UTR) of the transcript [2]. *pal-1* is the worm ortholog of *Drosophila caudal* (*cad*) and vertebrate *CDX* homeobox transcription factors, which are involved in posterior patterning and gut formation. In the flour beetle *Tribolium*, translation repression of *cad* is equally dependent on MEX-3, whose depletion leads to severe defects in the anterior part of the body [3]. Recently, human MEX3A was shown to play a similar function as its ortholog, but in the gastrointestinal context, where it downregulates the CDX2 intestinal transcription factor via MRE-dependent binding [4]. It is enticing to speculate that MEX3A might also regulate CDX2 during vertebrate embryonic development. Overall, these observations suggest that MEX-3 proteins might be part of an ancestral fate-promoting mechanism supporting anterior patterning in different organisms, partially through *pal-1/cad/CDX* repression.

Phylogenetic analysis suggests that an ancestral *mex-3* undergoes two rounds of duplication after vertebrate lineage divergence, accounting for the presence of four homologous genes in humans and mice (Figure 1A). These genes are denominated *MEX3A* to *MEX3D* and encode closely related phosphoproteins displaying differential expression patterns across human tissues [5]. They contain two K homology (KH) domains that provide RNA-binding capacity. In support of the notion that they bind RNA, MEX3A and MEX3B have been shown to localize to P bodies, which are recognized sites of mRNA turnover [4,5]. In addition to RNA binding, putative neo-functionalization might have evolved with the acquisition of a carboxy terminus really interesting new gene (RING) finger module that is lacking in *C. elegans* MEX-3. RING domain-containing proteins have been shown to mediate E3 ubiquitin ligase activity, and such a role has been demonstrated for human MEX3C, which promotes RING-dependent degradation of the human leukocyte antigen serotype A2 (*HLA-A2*) mRNA, consequently modulating natural killer cell activity (Figure 1B) [6]. The ability of the human MEX3 proteins to interact with distinct RNA sequences, together with mechanistic diversification potentiated by the RING domain, supports an evolutionarily conserved involvement in post-transcriptional regulation, although with escalating complexity and, thus far, little evidence for redundancy.

Different findings suggest a link between MEX3 proteins and disease, particularly cancer. In agreement with the master regulatory role of CDX2 in gastrointestinal homeostasis and carcinogenesis, impaired differentiation and altered polarity were observed when human MEX3A was overexpressed in an intestinal cell line, concomitant with gain of stemness features (Figure 1C) [4]. Interestingly, maintenance of active germ cell mitosis by MEX-3 in *C. elegans* comprises repression of the cell-cycle inhibitor cyclin-dependent kinase inhibitor-2 (CKI-2) [7], whose

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Keywords: MEX-3; RNA-binding protein; post-transcriptional regulation; CDX2; development; carcinogenesis.

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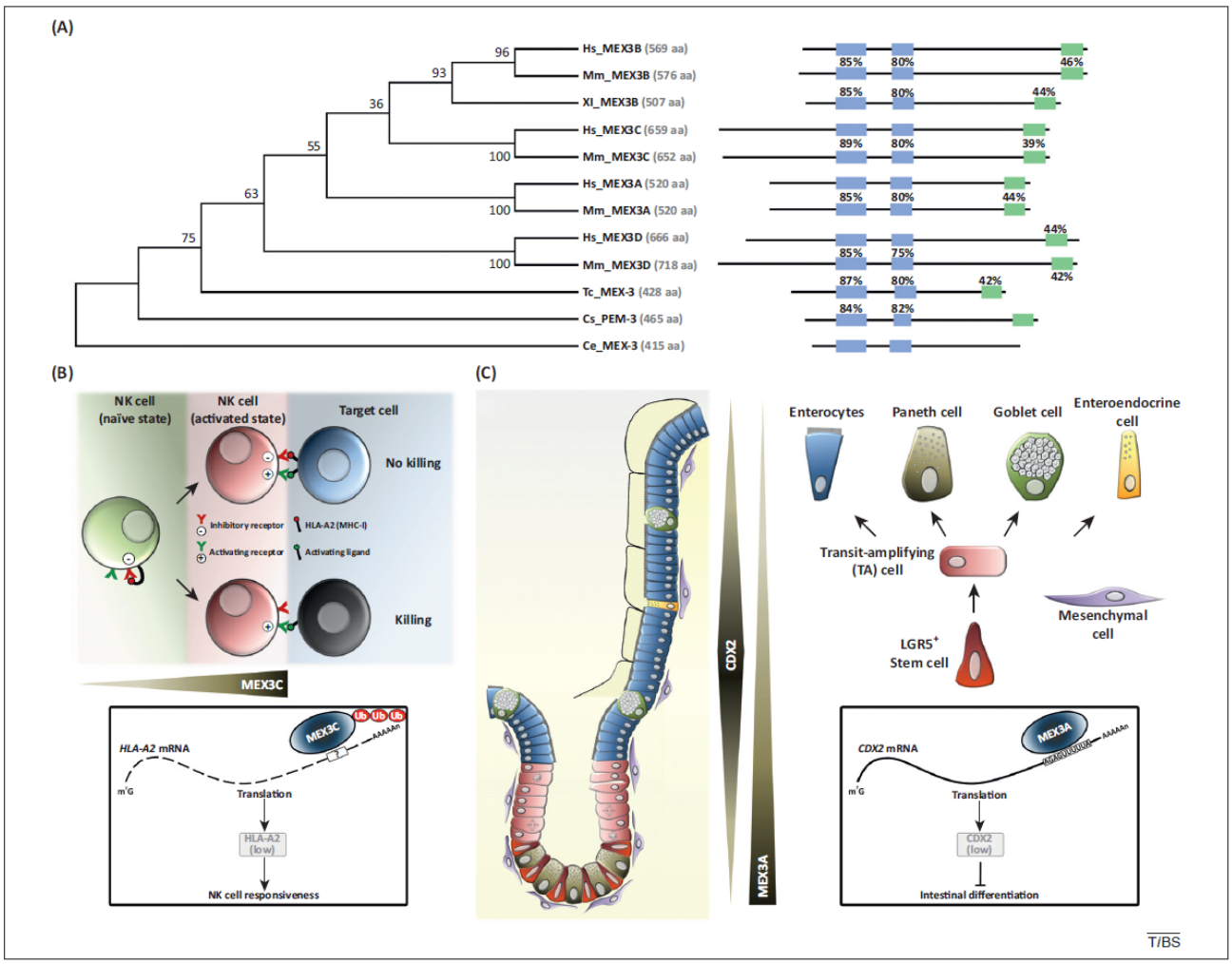

Figure 1. MEX-3 protein family evolutionary history and functions. **(A)** The following amino acid sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2): *Caenorhabditis elegans* (Ce)MEX-3 (NP_001021489.1); *Ciona savignyi* (Cs)PEM-3 (BAB03404.1); *Tribolium castaneum* (Tc)MEX-3 (NP_001137201.1); *Homo sapiens* (Hs)MEX3A (NP_001087194.1); *Mus musculus* (Mm)MEX3A (NP_001025061.2); HsMEX3B (NP_115622.2); MmMEX3B (NP_780575.2); *Xenopus laevis* (Xl)MEX3B (NP_001091216.1); HsMEX3C (NP_057710.3); MmMEX3C (NP_001034303.3); HsMEX3D (NP_001167589.1); MmMEX3D (NP_941017.2). The phylogenetic tree was inferred by the Maximum Likelihood method using MEGA5 software (http://www.megasoftware.net). The percentage of trees in which the associated taxa clustered together is shown next to the branches (1000 bootstrap replicates). A graphic of the different MEX-3 proteins is shown, depicting the identity percentage between the different K homology (KH) domains (blue boxes) in relation to the ones from CeMEX-3 and between the really interesting new gene (RING) domains (green boxes) in relation to the one from CsPEM-3. **(B)** MEX3C modulates natural killer (NK) cell activity. In naïve NK cells, MHC-I antigens such as HLA-A2 constitutively bind inhibitory receptors in *cis*, preventing spontaneous induction. In the activated condition, MEX3C expression levels increase, promoting RING-dependent degradation of the *HLA-A2* mRNA through ubiquitin E3 ligase activity and increasing NK cell responsiveness. **(C)** MEX3A impairs intestinal cell differentiation by interacting with a MEX-3 recognition element (MRE) present in the *CDX2* mRNA 3′ untranslated region (UTR) and repressing CDX2 protein expression. The MEX3A and CDX2 proteins show partial inversely correlated expression patterns along the vertical crypt–villus axis in mouse normal intestine.

human orthologs p21, p27, and p57 are known tumor suppressors. By contrast, *MEX3C* was described as a new chromosomal instability (CIN) suppressor in CIN$^+$ colorectal cancer [8]. Silencing of *MEX3C* induces DNA replication stress, structural chromosome abnormalities, and chromosome mis-segregation. Moreover, bioinformatics search of Oncomine, a cancer microarray data-mining platform (http://www.oncomine.com) further strengthens this link, because *MEX3* transcript levels are altered in several cancer types. Additional association with disease, namely, metabolic changes, has been suggested. Downregulation of murine *Mex3c* by gene trapping shows that it plays an important role in regulation of energy balance, because these mice have reduced adipose deposition and increased energy expenditure [9]. *Mex3c* mutant mice also display a postnatal growth retardation phenotype; possibly due to a role for MEX3C in enhancing insulin-like growth factor 1 (*Igf1*) mRNA translation in developing bone, given that IGF1 is a primary mediator of growth hormone effects. Finally, polymorphic variations in human *MEX3C* confer susceptibility to essential hypertension [10].

In conclusion, the available evidence implicates MEX-3 proteins in core biological processes, from embryonic development to epithelial homeostasis, immune responses, metabolism, and cancer. Outstanding challenges include: (i) identification of *bona fide* RNA targets and analysis of the mechanism underlying their recognition and regulation; (ii) determining the extent of functional redundancy among MEX-3 family members; (iii) assessing how they interact with signaling pathways and contribute to context-specific molecular processes; and (iv) analyzing their physiological and pathological roles. To achieve these goals

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and understand the *in vivo* relevance of MEX3 proteins may require the generation of genetically modified gain- and loss-of-function animal models that allow modulation of MEX3 levels and activity in a time- and tissue-specific manner, combined with crosslinking immunoprecipitation experiments and high-throughput sequencing (CLIP-Seq). Ascertaining their contribution to carcinogenesis and evaluating their potential as markers of cancer progression or prognosis is another important future goal. For the time being, the MEX-3 family of proteins constitutes a fascinating example of the way a patterning gene can adapt to function in complex and diverse fine-tuning regulatory mechanisms of different organisms – a cunning '*mex*ture' towards evolutionary success.

Acknowledgments

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References

1 Ciosk, R. *et al.* (2006) Translational regulators maintain totipotency in the *Caenorhabditis elegans* germline. *Science* 311, 851–853

2 Pagano, J. *et al.* (2009) RNA recognition by the embryonic cell fate determinant and germline totipotency factor MEX-3. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20252–20257

3 Schoppmeier, M. *et al.* (2009) An ancient anterior patterning system promotes caudal repression and head formation in ecdysozoa. *Curr. Biol.* 19, 1811–1815

4 Pereira, B. *et al.* (2013) CDX2 regulation by the RNA-binding protein MEX3A: impact on intestinal differentiation and stemness. *Nucleic Acids Res.* 41, 3986–3999

5 Buchet-Poyau, K. *et al.* (2007) Identification and characterization of human Mex-3 proteins, a novel family of evolutionarily conserved RNA-binding proteins differentially localized to processing bodies. *Nucleic Acids Res.* 35, 1289–1300

6 Cano, F. *et al.* (2012) The RNA-binding E3 ubiquitin ligase MEX-3C links ubiquitination with MHC-I mRNA degradation. *EMBO J.* 31, 3596–3606

7 Kalchhauser, I. *et al.* (2011) FBF represses the Cip/Kip cell-cycle inhibitor CKI-2 to promote self-renewal of germline stem cells in *C. elegans*. *EMBO J.* 30, 3823–3829

8 Burrell, R. *et al.* (2013) Replication stress links structural and numerical cancer chromosomal instability. *Nature* 494, 492–496

9 Jiao, Y. *et al.* (2012) Mex3c mutation reduces adiposity and increases energy expenditure. *Mol. Cell. Biol.* 32, 4350–4362

10 Guzmán, B. *et al.* (2006) Implication of chromosome 18 in hypertension by sibling pair and association analyses: putative involvement of the RKHD2 gene. *Hypertension* 48, 883–891

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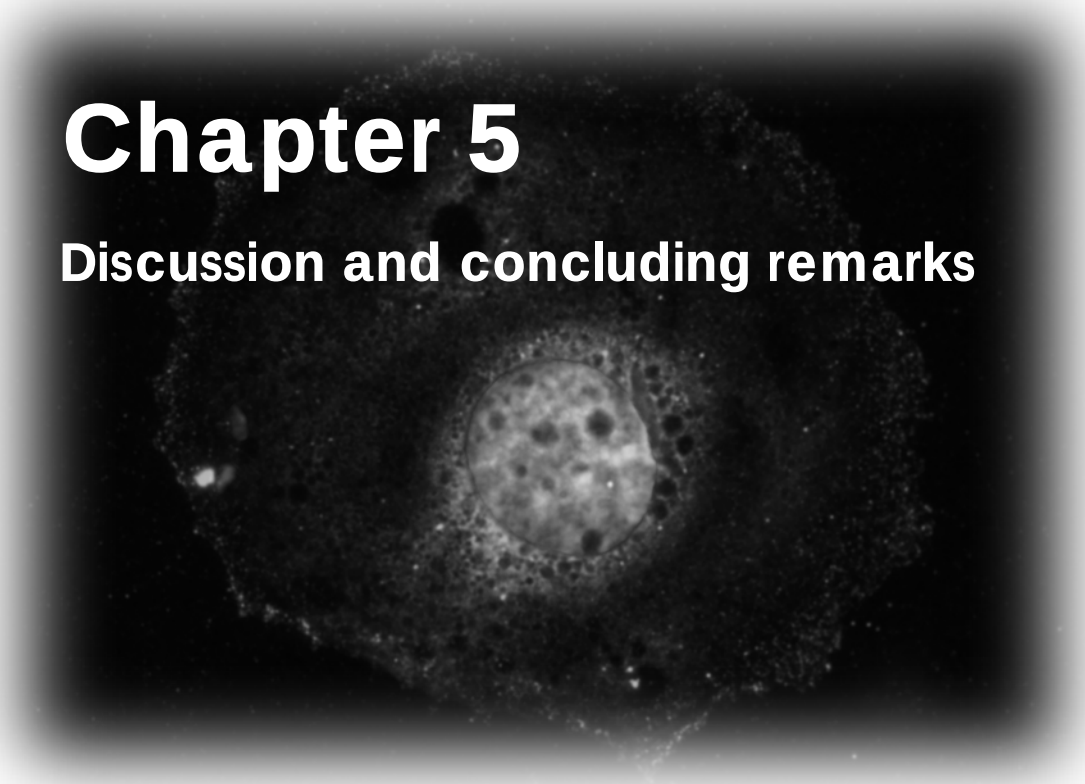
Chapter 5

Discussion and concluding remarks



GI malignancies remain a leading cause of morbidity worldwide, affecting almost four million individuals per year. This health burden has been intensively tackled during the last decades in the search for new approaches of prevention and management, and though slow, some progress has been achieved. A major advancement in understanding cancer aetiology is the recognition that although intrinsically genetic in origin, its heterogeneous nature stems from complex interactions between environmental and host factors. At the cellular level, this implies that cancer cells are not isolated entities solely defined by the linear accumulation of irreversible genetic alterations. Carcinogenesis is a rather dynamic process characterized by ongoing adaptations to the surrounding microenvironment.

The homeobox transcription factor CDX2 is a master regulator of intestinal differentiation. Not surprisingly, CDX2 deregulation is associated with GI carcinogenic processes, including IM onset during gastric cancer development and CRC, though its role in the latter is not consensual. Since structural alterations in the *CDX2* locus are rare, it has become evident that regulation must account for the major alterations reported in CDX2 levels in different pathological conditions and consequently, for maintaining its normal homeostatic balance. Henceforth, efforts have been concentrated in defining the regulatory mechanisms underlying CDX2 expression. Indeed, several transcriptional, post-transcriptional and post-translational mechanisms have been described to control CDX2, making this a highly complex and tightly organized regulatory system.

With the objective of unveiling new molecular mechanisms of CDX2 regulation, we focused on the role of microenvironmental influences as a guiding line. In this regard, we have studied the effect of methylation at the *CDX2* promoter level and of MEX3A protein over *CDX2* expression, an RNA-binding factor uncovered in a cell-matrix interaction model. The different parts of this work are now debated in distinct sections, being pointed out within each one future research avenues of interest. At the end, a general conclusion is provided, integrating all data from a molecular and evolutionary standpoint.

5.1 *CDX2* REGULATION BY PROMOTER METHYLATION

Our analysis focused on two classically defined CpG islands, identified in the 5`proximal flanking region, and thus, more likely involved in *CDX2* regulation given the transcription start site proximity. We initially chose a panel of gastric cancer cell lines displaying differential expression of *CDX2* mRNA to validate a bisulfite-modified DNA sequencing protocol and establish possible correlations with the *CDX2* gene methyl-pattern. The choice of strategy was based on the fact that methylation-specific PCR uses different pairs of primers to specifically amplify methylated or unmethylated sequences, providing a semi-quantitative output that might not be representative of the whole CpG island, while sequencing of individual PCR amplicons with an unbiased primer pair generates extensive methylation maps at single-nucleotide resolution. Herewith, we were able to determine that promoter methylation status does not correlate with *CDX2* expression levels, which has been confirmed in following studies (Varon *et al.* 2012; Zhang *et al.* 2013). This is in accordance with the *CDX2* methylation frequency not showing any relationship to different clinicopathological characteristics, like tumour histological type or invasion (Yuasa *et al.* 2005), a fact at odds with the knowledge that gastric cancers retaining CDX2 expression are less aggressive and more differentiated (Liu *et al.* 2007a). Contradictory results have also been published in oesophageal tissues, with the presence of methylated and unmethylated alleles in cancer, and unmethylated in matched normal epithelia, despite no detectable mRNA or protein in the latter (Vaninetti *et al.* 2009).

We were interested in assessing methylation in IM pre-malignant condition compared to the normal gastric mucosa. We noticed the upper CpG island was heavily methylated in all cell lines tested, consistent with a previous description (Kawai *et al.* 2005), and hence could not be discriminative. As a result, we limited the tissue analysis to the lower CpG island only. Analysis of normal colonic mucosa, IM foci and matched adjacent normal epithelium showed a methylation pattern deemed inconsistent with the *CDX2* tissue-type expression. Hence, *CDX2* expression in IM is not attributable to demethylation, but to transcriptional activation by other mechanisms. This lack of correlation has also been observed in stomachs from normal mice and IM cases from *Cdx2*-transgenic mice (Mutoh *et al.* 2009). Concerning the intestinal background, a recent report stated that *CDX2* promoter hypermethylation is rare in CRC cases and cell lines (Salari *et al.* 2012). Therefore, it

seems *CDX2* promoter methylation is not causative for its own levels in intestine either, and cannot account for the subset of CRCs presenting heterogeneous CDX2 expression.

Most studies, including our own, assumed that functionally relevant DNA methylation occurs in proximal promoter areas, but it is possible that other sites might have been neglected. In fact, bioinformatic analysis reveals that at least three additional CpG islands exist in more distal 5` intergenic locations, approximately 6kb, 8kb and 11kb from the *CDX2* transcriptional start site, the last two overlapping the neighbouring gene *PRHOXNB*. CpG shore methylation is an emerging concept that might also be worthwhile exploring in this context. These are regions of lower CpG density that lie in close proximity, but often not within, CpG islands. Notably, and at least for colon, most cancer-associated alterations in methylation were predominantly observed in these areas, rather than restricted to CpG islands (Irizarry *et al.* 2009). In principle, these putative methyl-sites should be responsive to inhibitors of DNA methyltransferase activity as well. Nevertheless, the broad range nature of this type of treatments is expected to generate indiscriminate effects on multiple targets, whose net balance might not reflect increased *CDX2* transcription. Thus, methylation profile analysis of these alternative regions stands as a future research goal. Another key point to be considered is the role of chromatin state. Cell type-specific chromatin organization enables differential access to and activity of regulatory elements and the manifestation of unique cellular phenotypes. Alterations in these signatures are a common feature of cancer initiation and development (Suvà *et al.* 2013). Active promoter regions include heightened nuclease sensitivity implying nucleosome depletion, and histone modifications associated with transcriptional activation, such as methylation of histone H3 at lysine 4 (H3K4me1 or H3K4me3) and histone H3 acetylated at lysine 27 (H3K27Ac). Quite the opposite, nucleosome compaction, methylation of histone H3 at lysine 9 (H3K9me2 or H3K9me3) and of histone H3 at lysine 27 (H3K27me3) are essentially repressive traits (Zhou *et al.* 2011). In this regard, chromatin changes associated with *CDX1* and *CDX2* expression were only described in one publication and in colon cancer cell lines (Lu *et al.* 2008). The *in vivo* pattern of these epigenetic marks, particularly in IM development, remains to be examined.

5.2 3D AGS CELL MODEL AND LOSS OF CDX2 EXPRESSION

Given that previous reports described the role of epithelial-mesenchymal crosstalk as relevant for *CDX2* expression, we established *in vitro* 3D culture models in order to study interactions between gastric epithelial cells and stromal elements that might regulate CDX2. These systems have now been applied with success to generate and maintain gastric and intestinal architecture in the form of organoids from single *Lgr5*$^{+}$ stem cells (Sato *et al.* 2009; Barker *et al.* 2010).

Our transcriptomic analysis comparing 3D with standard AGS cell culture revealed a remodeling of the gene expression program. Such adaptation was particularly noticeable by alterations in actin reorganization mediated by increased expression of GTPases of the Rho family, like *ARHGEF9*, which acts as a guanine nucleotide exchange factor for the polarity-related protein CDC42. An increase in *COL4A5* and *LAMB1* transcripts, for example, was also observed, which encode for type IV collagen and laminin, respectively, components of the basal lamina that has been shown to be primarily a product of epithelial cells, pointing towards an inductive feedback loop between the matrix and epithelial cells. Interestingly, we noticed a significant increase in cell cycle related elements, for instance in *SGOL1*, which encodes a protein that shields the cohesin complex from cleavage and is crucial for faithful chromosome segregation during mitosis and meiosis (Kahyo *et al.* 2011); *ERCC6L*, a DNA helicase that is essential for maintenance of genome integrity (Baumann *et al.* 2007); and the polymerase *POLD1*, which functions in DNA replication/repair and whose mutations affecting the proofreading domains have recently been shown to increase the risk for colorectal carcinoma (Palles *et al.* 2013). Transcripts encoding inflammatory response factors, like chemokine ligands, interferons and interleukins, were all downregulated. Furthermore, an increase in *Notch1*, *DLL1*, and *FZD1* was detected. Combinatorial control between these Notch and Wnt pathway members seems necessary to maintain intestinal stem cells (Fre *et al.* 2009). In line, the array showed a 60-fold increase in the expression of *OLFM4*, a specific marker of *Lgr5*$^{+}$ intestinal stem cells (van der Flier *et al.* 2009).

The previous results, together with the decreased CDX2 expression suggest that our AGS 3D cells resemble undifferentiated intestinal cells. Since AGS was first established from an intestinal-type gastric adenocarcinoma, we speculate that a partial reversion of the malignant towards an intestinal phenotype might occur under a physiologically more relevant microenvironment, reminiscent of what has been

observed in a mammary model (Weaver *et al.* 1997). The full potential of this model thus remains to be explored, as it might provide additional insights on factors involved in the regulation of intestinal differentiation.

5.3 MECHANISTIC ASPECTS OF MEX3A FUNCTION

Through microarray analysis to dissect the transcriptional regulatory circuitry of AGS cells in culture with an extracellular matrix, and further supported by an evolutionary link, we identified and validated MEX3A as a CDX2 repressor. Considering the distinct experimental settings established, our interpretation is that MEX3A actively controls CDX2 expression during proliferative states, when fine-tuning of CDX2 at the transcript level seems more relevant to couple cell cycle arrest with the emergence of a differentiation program (Bai *et al.* 2003). In subsequent differentiated states, CDX2 expression has already been demonstrated to rely mostly in its protein stability (Boulanger *et al.* 2005). Hence, MEX3A might exhibit target specificity, and this hypothesis is favoured by the fact that it is still detected in the differentiated section of the crypt-villus unit, albeit weaker than in the lower portions, arguing for some activity in this compartment that might not be CDX2-oriented.

Regarding the spatial compartmentalization in P bodies, we did not address if these are functionally relevant or if MEX3A effect on mRNA takes place as efficiently in the cytoplasm. Nevertheless, evolutionary comparative data supports this specific localization. *C. elegans* MEX-3 is involved in the correct segregation of large foci known as P granules to the final germline precursor (Draper *et al.* 1996; Schisa *et al.* 2001; Jud *et al.* 2007). P granules are membrane-free ribonucleoprotein cytoplasmic structures that primarily contain maternally expressed mRNAs alongside components shared with both P bodies and stress granules (Updike and Strome 2010). These three discrete microdomains are dynamically and reversibly induced in response to environmental signals. Thus, their nature seems to be in accordance with a function in maintaining or modifying certain RNA properties, delaying or even preventing the translational process. Inhibition of specific P body components through an RNAi strategy will help to shed light over MEX3A functional requirements. We showed that MEX3A interacts with *CDX2* mRNA through a canonical MRE present in the 3`UTR. This

does not exclude the possibility of MEX3A recognizing either different variations of the degenerate sequence described for MEX-3 binding in *C. elegans* (Pagano *et al.* 2009) or even other unknown sequences; however, it provides an anchor point to search for new targets. We have not assessed if the interaction is direct or mediated by another RBP that complexes with MEX3A, either through its RING domain or even through the KH domains, as these have also been shown to mediate protein oligomerization in some instances (Chen *et al.* 1997). RNA electrophoretic mobility gel shift assays with a probe containing the *CDX2* 3`UTR MRE sequence together with a MEX3A recombinant protein will provide a definitive answer concerning this issue. Another aspect that was not advanced is MEX3A own sequence and/or structural interaction determinants. MEX-3 proteins possess type I KH domains, which consist of three-stranded antiparallel β-sheets, oriented against three α-helices. The stable β-α-α-β-β-α conformation exposes a flexible loop between the first two helices, occupied by semi-conserved positively charged residues in a Gly-X-X-Gly motif (Valverde *et al.* 2008). Even though the total number of solved structures with

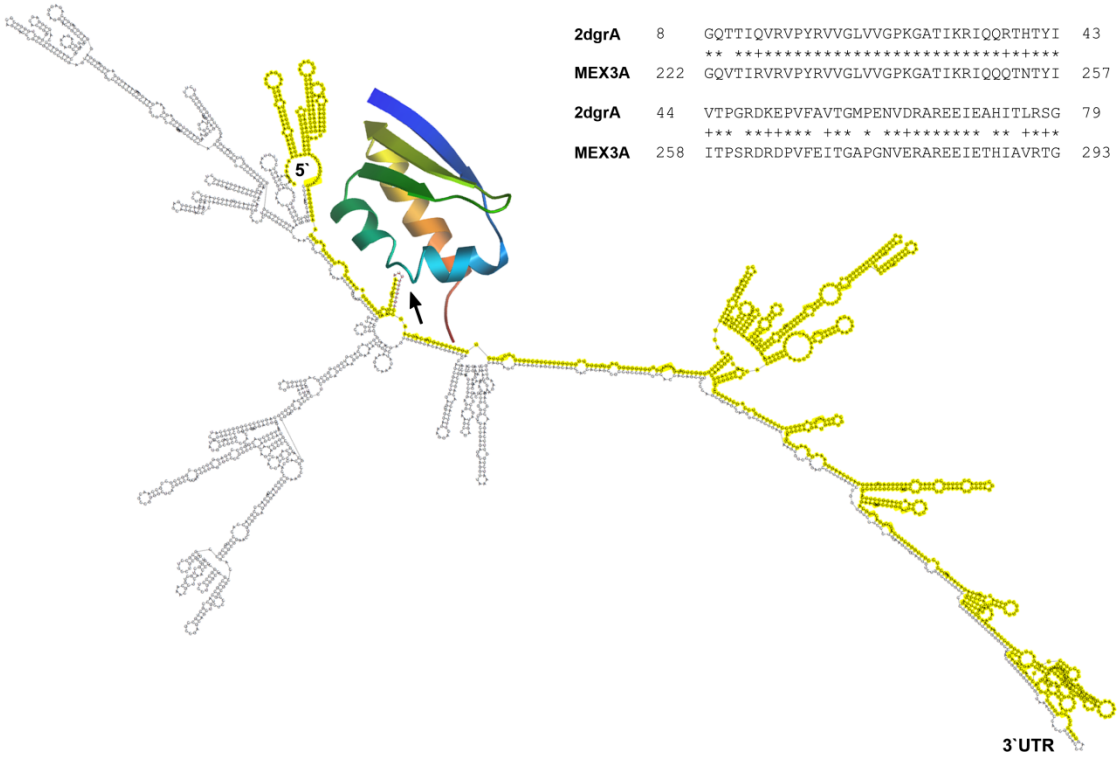


Figure 1. Bioinformatic structural analysis of MEX3A-*CDX2* interaction. The *CDX2* mRNA secondary structure was predicted using the RNAfold program default parameters (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The 3`UTR is highlighted in yellow (3`UTR), as well as the beginning of the first exon (5`). The MEX3A second KH domain structure was predicted with the SWISS-MODEL program (http://swissmodel.expasy.org/). The black arrow points towards the putative interaction site between the MRE in *CDX2* 3`UTR and the MEX3A second KH domain flexible loop containing the GXXG motif. The protein sequence alignment of the MEX3D second KH domain (2dgrA) and MEX3A second KH domain depicts a high degree of homology between both.

KH domains bound to cognate nucleic acid ligands is small, it seems this tetrapeptide is the putative RNA recognition surface. Data on the MEX3D second KH domain structure is available (Protein Data Bank entry 2dgrA), and since it shares 76% sequence identity with the corresponding MEX3A domain, the former can be used as template to model the second, providing insights into the possible docking process with target mRNAs (**Figure 1**). The KH domains role awaits experimental validation through the use of site-directed mutagenesis to introduce mutations in the conserved tetrapeptide and assess binding activity over *CDX2* mRNA as readout.

Most known examples of RBP action include proteins that repress translation, and under most circumstances initiation is the rate-limiting step, whose complexity and importance are underscored by the fact that it is assisted by more than 25 polypeptides, while only a limited set of factors are dedicated for elongation and termination (Gebauer and Hentze 2004). During initiation, the scaffold factor Eukaryotic initiation factor (eIF)4G interacts both with the cap-binding factor eIF4E and the poly(A) binding protein (PABP), which is in turn bound to the poly(A) tail, resulting in mRNA pseudo-circularization (Wells *et al.* 1998). This mRNA closed-loop arrangement thus provides a physical framework for the action of 3`UTR effectors on translation initiation at the 5`end. *Drosophila* Bicoid (Bcd), for instance, binds to the 3`UTR of *cad* mRNA and recruits an eIF4E-homologous protein (4EHP) to the cap structure, inhibiting translation because of its low affinity for eIF4G (Cho *et al.* 2005) [**Figure 2A**]. Other RBPs recruit factors that mimic eIF4G and compete with it for eIF4E-binding. Such is the case of *Xenopus* cytoplasmic polyadenylation element binding protein (CPEB), which binds the 3`UTR of *cyclin B1* mRNA and recruits the eIF4E-binding protein (4E-BP) Maskin to repress translation (Stebbins-Boaz *et al.* 1999) [**Figure 2B**]. But translation can also be controlled at late-initiation, in a cap-independent manner. During erythroid differentiation, hnRNPs K and E1 bind to the *15-Lipoxygenase* (*ALOX15*) mRNA 3`UTR and control translation by blocking the 60S large ribosomal subunit loading (Ostareck *et al.* 2001) [**Figure 2C**].

Based upon our results, it is likely that MEX3A is specifically affecting translation of the *CDX2* mRNA, by a mechanism similar to the ones described. An effect upon nuclear processing of the transcript is unlikely, as we have detected comparable levels of *CDX2* mRNA in transient overexpression experiments. Therefore, MEX3A might recruit factors that bind eIF4E and block its interaction with eIF4G (**Figure 2D, arrow 1**), it might interact directly with eIF4E or eIF4E-like isoforms (**Figure 2D. arrow 2**),

and/or it might hinder ribosomal subunit loading into the mRNA (**Figure 2D, arrow 3**). It has been shown that the interaction between eIF4E and either eIF4G or the 4E-BPs involves the sequence motif YXXXXLΦ, where X is any amino acid and Φ is hydrophobic (Rhoads 2009). Interestingly, an YRVVGLV motif is conserved in the MEX-3 family members. Although most RBPs target the initiation step of translation, some regulators have been recently reported to target the elongation step (Friend *et al.* 2012). Likewise, MEX3A might act at this point, interfering with the activity of specific eukaryotic translation elongation factors, which ultimately might lead to ribosome stalling (**Figure 2D, arrow 4**). It is also possible that some of these mechanisms require MEX3A functional interplay with miRs to reinforce repression (**Figure 2D, arrow 5**), a hypothesis further strengthened by the fact that *CDX2* is a target of specific miRs whose binding sites lie in close proximity to the MRE. Protein co- immunoprecipitation experiments against translation factors, together with ribosome-profiling assays to ascertain whether *CDX2* mRNA co-fractionates with polysomes in the MEX3A-overexpressing cells, will allow us to unveil the translation step and mechanism underlying the MEX3A regulatory function.

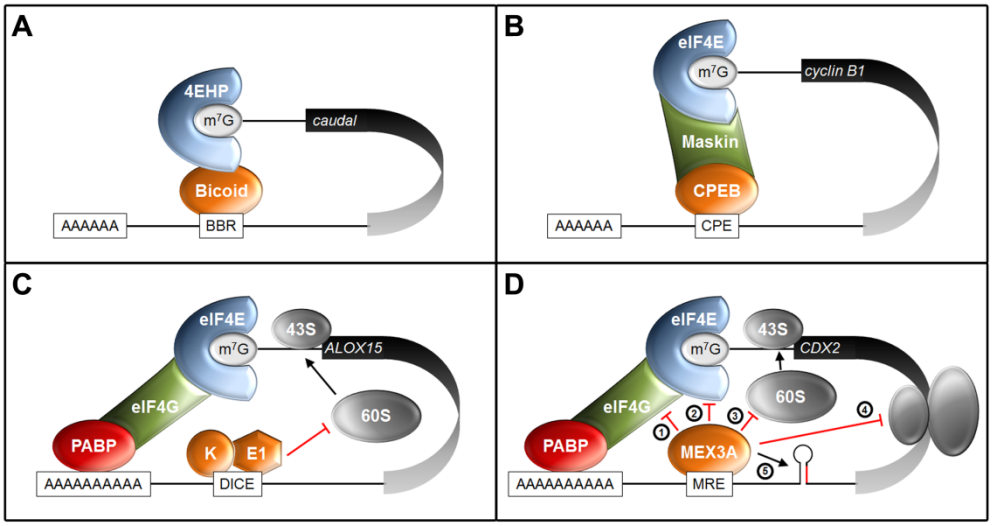


Figure 2. Mechanims of translational control via the 3`UTR. (**A**) *Drosophila* Bicoid inhibits *caudal* mRNA translation by binding simultaneously to the 3`UTR Bicoid-binding region (BBR) and the eIF4E-homologous protein 4E-HP. (**B**) Translation of *cyclin B1* is repressed by displacement of eIF4G by Maskin, recruited to the mRNA by the cytoplasmic polyadenylation element (CPE)-binding protein (CPEB). (**C**) Inhibition of 60S joining has been documented in the control of *15-Lipoxygenase* (*ALOX15*) mRNA translation in erythroid precursor cells by binding of hnRNPs K and E1 to the 3`UTR differentiation-control element (DICE). (**D**) The modes of translational regulation MEX3A might use to modulate CDX2 expression are indicated by numbered arrows.

5.4 MEX3A AND CARCINOGENESIS

When we initiated the study of the regulatory role of MEX3A over CDX2 expression, the available information about the MEX-3 family members was limited. It was only recently that several publications have shed light on the different biological processes in which the mammalian members are involved.

The *MEX3A* transcript was found to be upregulated in about 64% of gastric cancer tissues compared with matched adjacent non-cancerous tissues, arguing in favour of a role of MEX3A in gastric carcinogenesis (Jiang *et al.* 2012). It is possible that MEX3A is responsible for the decreased CDX2 expression observed in about half of the gastric cancer cases. Data collected from the Oncomine cancer microarray database (www.oncomine.com) provides preliminary evidence in support of this assumption, as an outlier analysis ranks *MEX3A* among the top 2% genes overexpressed in a subset of gastric adenocarcinomas among two independent datasets comprising a total of 243 cancer cases. On the other hand, it is unlikely that MEX3A altered levels are directly involved in *de novo* CDX2 expression associated with IM onset, as *CDX2* mRNA is absent from the normal gastric mucosa, hampering any type of post-transcriptional regulatory mechanism. Nevertheless, it will be interesting to determine the expression pattern of MEX3A protein in normal epithelium, IM, dysplasia and cancer tissues to begin elucidating its role in the gastric carcinogenic process. Our results bring forward a possible role for MEX3A in colorectal carcinogenesis, not only through CDX2 regulation, but also through its association with loss of polarity and gain of stemness properties. MEX3A might directly contribute to the decreased CDX2 expression observed in a subset of colorectal carcinomas, particularly those with minimal differentiation. Furthermore, a prominent feature of differentiated and polarized simple epithelia is the presence of junctional complexes at the boundaries between neighboring cells. A modest increase in E-cadherin expression (Lorentz *et al.* 1997) and enhanced E-cadherin trafficking activity to the membrane (Funakoshi *et al.* 2010) has been reported in CDX2-overexpressing cell lines, leading to a mature morphogenesis, associated with increased adhesive potential. We have shown that expression of MEX3A is associated with decreased E-cadherin expression in 3D cysts, which might result from a direct translational repression by MEX3A or indirectly from MEX3A-induced CDX2 suppression. Interestingly, the *CDH1* transcript encoding E-cadherin harbors several MRE motifs in its 3`UTR. On the other hand, it has been shown that $LGR5^{+}$ crypt

intestinal stem cells are the cell of origin of adenomas (Barker *et al.* 2009) and that 74-85% of CRCs express a *Lgr5*$^{+}$ associated signature (Ziskin *et al.* 2013). These observations suggest that a stem cell/progenitor cell hierarchy is maintained in early stem-cell-derived adenomas and during cancer progression, which would lend support to the newly introduced cancer stem cell concept (Huang and Wicha 2008; Merlos-Suárez *et al.* 2011). We have shown an increase in intestinal stem cell markers expression, including *LGR5*, in MEX3A-overexpressing cells. Data extracted from Oncomine reveals an over 3-fold increase in *MEX3A* expression in two independent datasets comprising a total of 231 colorectal adenocarcinomas (**Figure 3**). Moreover, the RNA-binding QKI protein, the mammalian orthologue of GLD-1 and a key regulator of cell-cycle withdrawal and differentiation of the gastrointestinal epithelium, is downregulated in CRCs (Yang *et al.* 2010). Enticingly, GLD-1 is capable of repressing MEX-3 expression in *C. elegans* (Mootz *et al.* 2004). All these evidences favour a link between MEX3A and colorectal malignancy, which we will address in the near future.

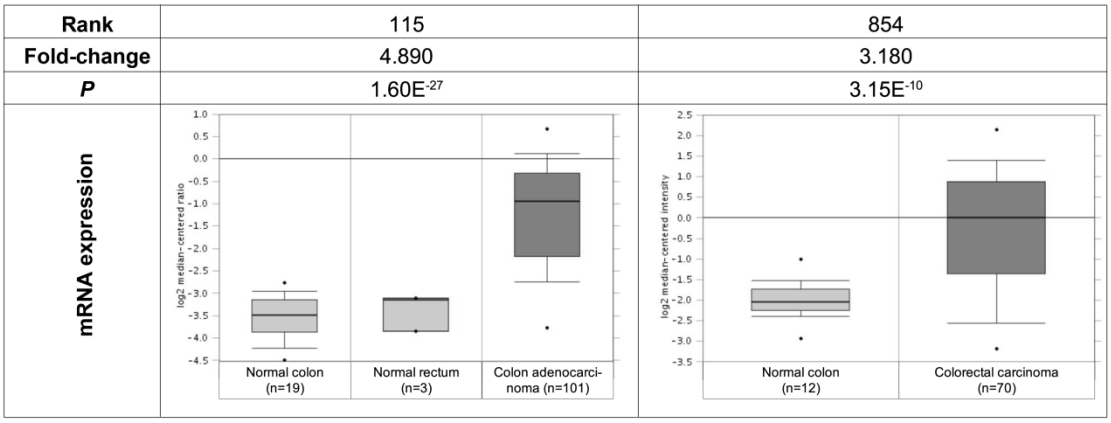


Figure 3. MEX3A expression in colorectal cancer. Screenshots of box-plot analysis, displaying the log2 median-centered expression profile of *MEX3A* transcripts in cancer and normal counterparts. The TCGA and Hong colorectal datasets were chosen using top 1% gene rank, fold-change $\geq$ 1.5 and *P* value (Student`s *t*-test) $\leq$ 1.00E^{-4} as thresholds.

The interplay we uncovered between MEX3A and stem cell properties is an aspect that parallels the role of *C. elegans* MEX-3 in keeping distal germ cells totipotency (Ciosk *et al.* 2006). In the most distal end of the nematode gonad, which can be viewed as a stem cell niche where active division occurs, MEX-3 functions redundantly with the PUF family protein PUF-8 to prevent mitotic germ cells from terminally differentiating (Ariz *et al.* 2009). Since *puf-8;mex-3* double mutant gonads maintain germ cell identity and differentiation capacity but have several mitotic

defects, germline-specific cell cycle regulators might constitute a potential group of PUF-8 and MEX-3 common targets. Other reports in different biological contexts secondarily reinforce this relationship. For example, it was shown that LIN28B inhibits the let-7 miR family and is capable of reprogramming hematopoietic progenitor cells from adult mice bone marrow, endowing them with the ability to mediate multi-lineage reconstitution that resembles foetal lymphopoiesis (Yuan *et al.* 2012). A deep RNA-sequencing approach identified a specific global derepression of putative let-7 target mRNAs that includes *Mex3a*. In addition, global gene expression profiles comparing the transition from quiescent to activated satellite muscle cells in response to injury show a global alteration in RBPs and miRs expression levels, including a decrease in *Mex3a* and *Mex3b* transcripts (Pallafacchina *et al.* 2010; Crist *et al.* 2012). RBPs might be critical to prevent uncontrolled growth and differentiation in any stem cell niche, be it embryonic or adult, where regulation of gene expression at the post-transcriptional level is a common theme (Sampath *et al.* 2008; Ghosh *et al.* 2009). Hence, it seems likely that MEX-3 proteins are important players in the reprogramming or maintenance of cells with multiple differentiation potentialities, by controlling expression of stem cell factors, suppressing differentiation, or both.

5.5 A CONSERVED FUNCTION FOR MEX3A IN MAMMALIAN EMBRYOGENESIS?

MEX-3 regulates blastomere identity during *C. elegans* embryogenesis, ensuring translational control of *pal-1* expression. But CDX2 is also an important mediator of trophectoderm (TE) cell fate specification in mammals. Given the high degree of homology between the MEX-3 family members, a role during mammalian embryogenesis might have been equally conserved.

The first report of a putative *mex-3* orthologue was on an invertebrate maternal transcript named posterior end mark 3 (*pem-3*), isolated from the ascidian *Ciona savignyi* (Satou 1999). The PEM-3 protein contains two KH domains which are 83% identical to the ones of *C. elegans* MEX-3 and an additional RING finger consensus sequence, but its function is unknown. As for the zygotic transcript, it was suggested to play a role in brain differentiation of the ascidian larvae. *Drosophila* Cad protein

plays a role in posterior patterning, accumulating in a posterior-to-anterior concentration gradient, even though *cad* mRNA is uniformly distributed in the syncytial embryo (Mlodzik *et al.* 1985). Interestingly, a MEX-3 orthologue is also present in *Drosophila*, however, it is not expressed at the blastoderm stage when the Cad gradient forms (Schoppmeier *et al.* 2009). It seems that, in this case, *cad* regulation has been taken over by Bcd, the primary determinant of anterior patterning (Driever and Nüsslein-Volhard 1988; Niessing *et al.* 2002). Surprisingly, the *bcd* gene possesses a limited phylogenetic distribution and has not been isolated from species other than Diptera (Brown *et al.* 2001). In the flour beetle *Tribolium*, a non-dipteran insect, it was observed that strong depletion of *Tc-mex-3* led to severe phenotypic aberrations, ultimately resulting in complete deletion of the entire head (Schoppmeier *et al.* 2009). The functional similarity of MEX-3 in the nematode, ascidian and beetle embryos suggests that MEX-3 is a basic element of an ancestral fate-promoting mechanism that supports anterior patterning, at least in part by *cad* repression.

As for mammalian embryonic development, one of the earliest molecular events observed during lineage determination is CDX2 expression in TE precursors (Beck *et al.* 1995). Lack of *Cdx2* in homozygous-null mouse embryos is associated with failure to downregulate *Oct4* and *Nanog* in outside cells, resulting in peri-implantation lethality (Niwa *et al.* 2005; Strumpf *et al.* 2005). Currently, two opposing lines of thought exist concerning CDX2 role during embryogenesis. On one side, *Cdx2* is regarded as not being essential for initiation of TE formation, acting only later during maintenance of TE (Ralston and Rossant 2008; Wu *et al.* 2010). On the other hand, a mutually reinforcing relationship between cellular polarity and expression of CDX2 has been advocated, such that positioning of cells influence expression of *Cdx2* and early CDX2 levels influence the proportion of blastomeres contributing to TE (Jedrusik *et al.* 2008; Jedrusik *et al.* 2010). Although the exact position of CDX2 in the lineage-determining hierarchy is not entirely clear, its tightly controlled spatio-temporal expression raises the important question of what are the molecular mechanisms acting underneath. *Cdx2* mRNA has been detected at low levels in oocytes, zygotes and in two- to eight-cell embryos (Wang *et al.* 2004b; Jedrusik *et al.* 2010; Wu *et al.* 2010). While the major burst of zygotic genome activation occurs at the late two-cell stage, *Cdx2* transcript levels only increase strikingly at the sixteen-cell stage. Moreover, CDX2 protein is clearly detected in blastomeres nuclei at the

eight-cell stage, which precedes establishment of distinct cell populations (Ralston and Rossant 2008; Jedrusik *et al.* 2010). Since these cells will give rise to both the inner cell mass and TE, the corollary of this delay is that *Cdx2* must be repressed during initial apolar divisions, and upon induction it must be silenced and maintained as cells are allocated to inside or outside positions, respectively. Transcriptional control in response to lineage segregation is an already established means of regulating *Cdx2* (Nishioka *et al.* 2009), but it is not well understood how this is imposed in the first place.

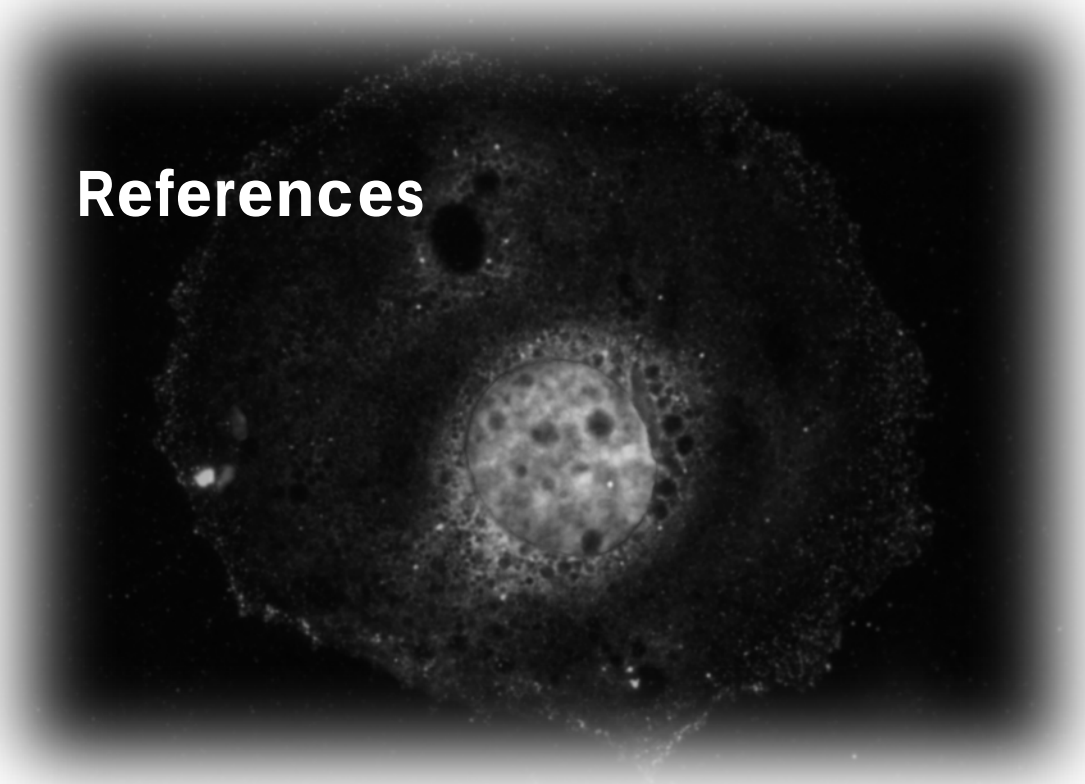
Asymmetric cell divisions produce daughter cells with distinct fates, and rely on the distinct segregation of key determinants, including localized mRNAs. Accordingly, it was reported that *Cdx2* transcripts become preferentially localized apically at the late eight-cell stage and asymmetrically inherited during mouse development at the eight- to sixteen-cell stage transition (Jedrusik *et al.* 2008). This was not seen for transcripts encoding other cell commitment transcription factors, like *Nanog*. A compelling hypothesis is that MEX3A might contribute to the initial *Cdx2* mRNA repression and possibly to its apical enrichment, recapitulating *C. elegans* MEX-3 effect over *pal-1*. This possibility is strengthened by a report showing that in mouse *Cdx2* asymmetric localization depends on a minimal *cis*-element comprising the last 97 nucleotides of the transcript open reading frame (Skamagki *et al.* 2013), where we had already identified one MRE and another one close upstream. Assuming this scenario, MEX3A might be involved in the regulation of the *Cdx2* transcript translation in an embryonic setting or even in its transport, as it was demonstrated that intact microtubules and actin cytoskeleton, together with the activity of motor proteins of the kinesin superfamily are a requirement for this asymmetric localization (Skamagki *et al.* 2013). Thus, MEX3 proteins might act as conserved components of the cell polarization pathway contributing to CDX2 regulation during mammalian embryogenesis, thereby linking gene regulation and positional information.

5.6 CONCLUDING REMARKS

With this work, we have advanced the knowledge on the molecular mechanisms of CDX2 regulation. On one hand, our results support the lack of evidence in favor of a role of methylation on *de novo CDX2* expression in the gastritis-metaplasia-carcinoma sequence, contributing to solve an ambiguous topic in the field. On the other hand, we provide evidence of a new mechanism of CDX2 post-transcriptional regulation mediated by the RNA-binding protein MEX3A. The relevance of MEX3A just started to be unraveled, but our data points towards its involvement in intestinal homeostasis and possibly gastrointestinal carcinogenesis, with effects in cellular polarity and stemness. In this regard, we will now ascertain the role of MEX3A by studying its expression in preneoplastic and neoplastic lesions, as well as its correlation with CDX2 and clinicopathological parameters. In parallel, we will establish a transgenic mouse model with conditional and inducible MEX3A expression in the villi compartment of the intestine, in order to assess the effects of ectopic MEX3A over the intestinal phenotype. This will be complemented by a transcriptome-wide analysis of MEX3A regulatory interactions. Overall, we expect to significantly contribute to the understanding of the impact of MEX3A post-transcriptional control on gene expression, both in homeostasis and disease.

Even considering MEX-3 well-described role in *C. elegans* embryonic development and maintenance of cell totipotency, it is remarkable to find such a marked degree of functional parallelism in higher organisms, a feature that one can predict to reflect the importance of this protein family. Consequently, it seems we have disclosed a case of evolutionary conservation that might have major implications for basic and clinical research.

References



Aliaga JC, Deschênes C, Beaulieu JF, Calvo EL, Rivard N. 1999. Requirement of the MAP kinase cascade for cell cycle progression and differentiation of human intestinal cells. *Am J Physiol* **277**: G631-641.

Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang J, De Bolós C, David L. 2003. Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. *J Pathol* **199**: 36-40.

Anantharaman V, Koonin E, Aravind L. 2002. Comparative genomics and evolution of proteins involved in RNA metabolism. *Nucleic Acids Res* **30**: 1427-1464.

Anderson P. 2008. Post-transcriptional control of cytokine production. *Nat Immunol* **9**: 353-359.

Anderson P, Kedersha N. 2006. RNA granules. *J Cell Biol* **172**: 803-808.

Aoki K, Kakizaki F, Sakashita H, Manabe T, Aoki M, Taketo MM. 2011. Suppression of colonic polyposis by homeoprotein CDX2 through its nontranscriptional function that stabilizes p27Kip1. *Cancer Res* **71**: 593-602.

Aoki K, Tamai Y, Horiike S, Oshima M, Taketo MM. 2003. Colonic polyposis caused by mTOR-mediated chromosomal instability in Apc+/Delta716 Cdx2+/- compound mutant mice. *Nat Genet* **35**: 323-330.

Ariz M, Mainpal R, Subramaniam K. 2009. C. elegans RNA-binding proteins PUF-8 and MEX-3 function redundantly to promote germline stem cell mitosis. *Dev Biol* **326**: 295-304.

Asonuma S, Imatani A, Asano N, Oikawa T, Konishi H, Iijima K, Koike T, Ohara S, Shimosegawa T. 2009. Helicobacter pylori induces gastric mucosal intestinal metaplasia through the inhibition of interleukin-4-mediated HMG box protein Sox2 expression. *Am J Physiol Gastrointest Liver Physiol* **297**: G312-322.

Bai YQ, Miyake S, Iwai T, Yuasa Y. 2003. CDX2, a homeobox transcription factor, upregulates transcription of the p21/WAF1/CIP1 gene. *Oncogene* **22**: 7942-7949.

Bai YQ, Yamamoto H, Akiyama Y, Tanaka H, Takizawa T, Koike M, Kenji Yagi O, Saitoh K, Takeshita K, Iwai T *et al.* 2002. Ectopic expression of homeodomain protein CDX2 in intestinal metaplasia and carcinomas of the stomach. *Cancer Lett* **176**: 47-55.

Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M *et al.* 2010. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* **6**: 25-36.

Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, Danenberg E, Clarke AR, Sansom OJ, Clevers H. 2009. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* **457**: 608-611.

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ *et al.* 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**: 1003-1007.

Barker N, van Oudenaarden A, Clevers H. 2012. Identifying the stem cell of the intestinal crypt: strategies and pitfalls. *Cell Stem Cell* **11**: 452-460.

Barros R, da Costa LT, Pinto-de-Sousa J, Duluc I, Freund JN, David L, Almeida R. 2011. CDX2 autoregulation in human intestinal metaplasia of the stomach: impact on the stability of the phenotype. *Gut* **60**: 290-298.

Barros R, Freund JN, David L, Almeida R. 2012. Gastric intestinal metaplasia revisited: function and regulation of CDX2. *Trends Mol Med* **18**: 555-563.

Barros R, Marcos N, Reis CA, De Luca A, David L, Almeida R. 2009a. CDX2 expression is induced by Helicobacter pylori in AGS cells. *Scand J Gastroenterol* **44**: 124-125.

Barros R, Mendes N, Howe JR, Reis CA, de Bolos C, Carneiro F, David L, Almeida R. 2009b. Juvenile polyps have gastric differentiation with MUC5AC expression and downregulation of CDX2 and SMAD4. *Histochem Cell Biol* **131**: 765-772.

Barros R, Peleteiro B, Almeida R, Figueiredo C, Barros H, David L, Lunet N. 2010. Relevance of high virulence Helicobacter pylori strains and futility of CDX2 expression for predicting intestinal metaplasia after eradication of infection. *Scand J Gastroenterol* **45**: 828-834.

Barros R, Pereira B, Duluc I, Azevedo M, Mendes N, Camilo V, Jacobs RJ, Paulo P, Santos-Silva F, van Seuningen I *et al.* 2008. Key elements of the BMP/SMAD pathway co-localize with CDX2 in intestinal metaplasia and regulate CDX2 expression in human gastric cell lines. *J Pathol* **215**: 411-420.

Baumann C, Körner R, Hofmann K, Nigg EA. 2007. PICH, a centromere-associated SNF2 family ATPase, is regulated by Plk1 and required for the spindle checkpoint. *Cell* **128**: 101-114.

Beck F, Chawengsaksophak K, Luckett J, Giblett S, Tucci J, Brown J, Poulsom R, Jeffery R, Wright NA. 2003. A study of regional gut endoderm potency by analysis of Cdx2 null mutant chimaeric mice. *Dev Biol* **255**: 399-406.

Beck F, Chawengsaksophak K, Waring P, Playford RJ, Furness JB. 1999. Reprogramming of intestinal differentiation and intercalary regeneration in Cdx2 mutant mice. *Proc Natl Acad Sci U S A* **96**: 7318-7323.

Beck F, Erler T, Russell A, James R. 1995. Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev Dyn* **204**: 219-227.

Benahmed F, Gross I, Gaunt SJ, Beck F, Jehan F, Domon-Dell C, Martin E, Kedinger M, Freund JN, Duluc I. 2008. Multiple regulatory regions control the complex

expression pattern of the mouse Cdx2 homeobox gene. *Gastroenterology* **135**: 1238.

Benahmed F, Gross I, Guenot D, Jehan F, Martin E, Domon-Dell C, Brabletz T, Kedinger M, Freund JN, Duluc I. 2007. The microenvironment controls CDX2 homeobox gene expression in colorectal cancer cells. *Am J Pathol* **170**: 733-744.

Bjerknes M, Cheng H. 1999. Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* **116**: 7-14.

Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, Clevers H, Jay P. 2004. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J Cell Biol* **166**: 37-47.

Bleuming SA, Kodach LL, Garcia Leon MJ, Richel DJ, Peppelenbosch MP, Reitsma PH, Hardwick JC, van den Brink GR. 2006. Altered bone morphogenetic protein signalling in the Helicobacter pylori-infected stomach. *J Pathol* **209**: 190-197.

Bonhomme C, Calon A, Martin E, Robine S, Neuville A, Kedinger M, Domon-Dell C, Duluc I, Freund JN. 2008. Cdx1, a dispensable homeobox gene for gut development with limited effect in intestinal cancer. *Oncogene* **27**: 4497-4502.

Bonhomme C, Duluc I, Martin E, Chawengsaksophak K, Chenard MP, Kedinger M, Beck F, Freund JN, Domon-Dell C. 2003. The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. *Gut* **52**: 1465-1471.

Bornschein J, Wex T, Peitz U, Kuester D, Roessner A, Malfertheiner P. 2009. The combined presence of H pylori infection and gastro-oesophageal reflux disease leads to an up-regulation of CDX2 gene expression in antrum and cardia. *J Clin Pathol* **62**: 254-259.

Bosman FT, Carneiro F, Hruban RH, Theise ND. 2010. *WHO classification of tumours of the digestive system, 4th edition*. IARC Press, Lyon.

Boudreau F, Rings EH, van Wering HM, Kim RK, Swain GP, Krasinski SD, Moffett J, Grand RJ, Suh ER, Traber PG. 2002. Hepatocyte nuclear factor-1 alpha, GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription. Implication for the developmental regulation of the sucrase-isomaltase gene. *J Biol Chem* **277**: 31909-31917.

Boulanger J, Vézina A, Mongrain S, Boudreau F, Perreault N, Auclair BA, Lainé J, Asselin C, Rivard N. 2005. Cdk2-dependent phosphorylation of homeobox transcription factor CDX2 regulates its nuclear translocation and proteasome-mediated degradation in human intestinal epithelial cells. *J Biol Chem* **280**: 18095-18107.

Boyd M, Hansen M, Jensen TG, Perearnau A, Olsen AK, Bram LL, Bak M, Tommerup N, Olsen J, Troelsen JT. 2010. Genome-wide analysis of CDX2 binding in intestinal epithelial cells (Caco-2). *J Biol Chem* **285**: 25115-25125.

Brabletz T, Spaderna S, Kolb J, Hlubek F, Faller G, Bruns CJ, Jung A, Nentwich J, Duluc I, Domon-Dell C *et al.* 2004. Down-regulation of the homeodomain factor Cdx2 in colorectal cancer by collagen type I: an active role for the tumor environment in malignant tumor progression. *Cancer Res* **64**: 6973-6977.

Bravo JC, Correa P. 1999. Sulphomucins favour adhesion of Helicobacter pylori to metaplastic gastric mucosa. *J Clin Pathol* **52**: 137-140.

Brown S, Fellers J, Shippy T, Denell R, Stauber M, Schmidt-Ott U. 2001. A strategy for mapping bicoid on the phylogenetic tree. *Curr Biol* **11**: R43-44.

Bry L, Falk P, Huttner K, Ouellette A, Midtvedt T, Gordon JI. 1994. Paneth cell differentiation in the developing intestine of normal and transgenic mice. *Proc Natl Acad Sci U S A* **91**: 10335-10339.

Buchet-Poyau K, Courchet J, Le Hir H, Séraphin B, Scoazec JY, Duret L, Domon-Dell C, Freund JN, Billaud M. 2007. Identification and characterization of human Mex-3 proteins, a novel family of evolutionarily conserved RNA-binding proteins differentially localized to processing bodies. *Nucleic Acids Res* **35**: 1289-1300.

Busà R, Paronetto MP, Farini D, Pierantozzi E, Botti F, Angelini DF, Attisani F, Vespasiani G, Sette C. 2007. The RNA-binding protein Sam68 contributes to proliferation and survival of human prostate cancer cells. *Oncogene* **26**: 4372-4382.

Camilo V, Barros R, Sousa S, Magalhães AM, Lopes T, Mário Santos A, Pereira T, Figueiredo C, David L, Almeida R. 2012. Helicobacter pylori and the BMP pathway regulate CDX2 and SOX2 expression in gastric cells. *Carcinogenesis* **33**: 1985-1992.

Chawengsaksophak K, James R, Hammond VE, Köntgen F, Beck F. 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* **386**: 84-87.

Chen AJ, Paik JH, Zhang H, Shukla SA, Mortensen R, Hu J, Ying H, Hu B, Hurt J, Farny N *et al.* 2012. STAR RNA-binding protein Quaking suppresses cancer via stabilization of specific miRNA. *Genes Dev* **26**: 1459-1472.

Chen T, Damaj BB, Herrera C, Lasko P, Richard S. 1997. Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1, and Qk1: role of the KH domain. *Mol Cell Biol* **17**: 5707-5718.

Cheng H, Leblond CP. 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* **141**: 537-561.

Cho PF, Poulin F, Cho-Park YA, Cho-Park IB, Chicoine JD, Lasko P, Sonenberg N. 2005. A new paradigm for translational control: inhibition via 5'-3' mRNA tethering by Bicoid and the eIF4E cognate 4EHP. *Cell* **121**: 411-423.

Chun SY, Chen F, Washburn JG, MacDonald JW, Innes KL, Zhao R, Cruz-Correa MR, Dang LH, Dang DT. 2007. CDX2 promotes anchorage-independent growth by transcriptional repression of IGFBP-3. *Oncogene* **26**: 4725-4729.

Ciosk R, DePalma M, Priess JR. 2006. Translational regulators maintain totipotency in the Caenorhabditis elegans germline. *Science* **311**: 851-853.

Correa P. 1992. Human gastric carcinogenesis: a multistep and multifactorial process—first American Cancer Society award lecture on cancer epidemiology and prevention. *Cancer Res* **52**: 6735-6740.

Coskun M, Boyd M, Olsen J, Troelsen JT. 2010. Control of intestinal promoter activity of the cellular migratory regulator gene ELMO3 by CDX2 and SP1. *J Cell Biochem* **109**: 1118-1128.

Coskun M, Olsen AK, Holm TL, Kvist PH, Nielsen OH, Riis LB, Olsen J, Troelsen JT. 2012. TNF-α-induced down-regulation of CDX2 suppresses MEP1A expression in colitis. *Biochim Biophys Acta* **1822**: 843-851.

Courchet J, Buchet-Poyau K, Potemski A, Brès A, Jariel-Encontre I, Billaud M. 2008. Interaction with 14-3-3 adaptors regulates the sorting of hMex-3B RNA-binding protein to distinct classes of RNA granules. *J Biol Chem* **283**: 32131-32142.

Crissey MA, Guo RJ, Fogt F, Li H, Katz JP, Silberg DG, Suh ER, Lynch JP. 2008. The homeodomain transcription factor Cdx1 does not behave as an oncogene in normal mouse intestine. *Neoplasia* **10**: 8-19.

Crissey MA, Guo RJ, Funakoshi S, Kong J, Liu J, Lynch JP. 2011. Cdx2 levels modulate intestinal epithelium maturity and Paneth cell development. *Gastroenterology* **140**: 517-528.

Crist CG, Montarras D, Buckingham M. 2012. Muscle satellite cells are primed for myogenesis but maintain quiescence with sequestration of Myf5 mRNA targeted by microRNA-31 in mRNP granules. *Cell Stem Cell* **11**: 118-126.

Crosnier C, Stamataki D, Lewis J. 2006. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet* **7**: 349-359.

da Costa LT, He TC, Yu J, Sparks AB, Morin PJ, Polyak K, Laken S, Vogelstein B, Kinzler KW. 1999. CDX2 is mutated in a colorectal cancer with normal APC/beta-catenin signaling. *Oncogene* **18**: 5010-5014.

Dang LH, Chen F, Ying C, Chun SY, Knock SA, Appelman HD, Dang DT. 2006. CDX2 has tumorigenic potential in the human colon cancer cell lines LOVO and SW48. *Oncogene* **25**: 2264-2272.

de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. 2009. Global signatures of protein and mRNA expression levels. *Mol Biosyst* **5**: 1512-1526.

de Vries AC, Meijer GA, Looman CW, Casparie MK, Hansen BE, van Grieken NC, Kuipers EJ. 2007. Epidemiological trends of pre-malignant gastric lesions: a long-term nationwide study in the Netherlands. *Gut* **56**: 1665-1670.

Debruyne PR, Witek M, Gong L, Birbe R, Chervoneva I, Jin T, Domon-Cell C, Palazzo JP, Freund JN, Li P *et al.* 2006. Bile acids induce ectopic expression of intestinal

guanylyl cyclase C Through nuclear factor-kappaB and Cdx2 in human esophageal cells. *Gastroenterology* **130**: 1191-1206.

Dicken BJ, Bigam DL, Cass C, Mackey JR, Joy AA, Hamilton SM. 2005. Gastric adenocarcinoma: review and considerations for future directions. *Ann Surg* **241**: 27-39.

Donnini M, Lapucci A, Papucci L, Witort E, Jacquier A, Brewer G, Nicolin A, Capaccioli S, Schiavone N. 2004. Identification of TINO: a new evolutionarily conserved BCL-2 AU-rich element RNA-binding protein. *J Biol Chem* **279**: 20154-20166.

Draper BW, Mello CC, Bowerman B, Hardin J, Priess JR. 1996. MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. *Cell* **87**: 205-216.

Driever W, Nüsslein-Volhard C. 1988. The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. *Cell* **54**: 95-104.

Drummond F, Putt W, Fox M, Edwards YH. 1997. Cloning and chromosome assignment of the human CDX2 gene. *Ann Hum Genet* **61**: 393-400.

Drummond F, Sowden J, Morrison K, Edwards YH. 1996. The caudal-type homeobox protein Cdx-2 binds to the colon promoter of the carbonic anhydrase 1 gene. *Eur J Biochem* **236**: 670-681.

Duluc I, Lorentz O, Fritsch C, Leberquier C, Kedinger M, Freund JN. 1997. Changing intestinal connective tissue interactions alters homeobox gene expression in epithelial cells. *J Cell Sci* **110**: 1317-1324.

Duprey P, Chowdhury K, Dressler GR, Balling R, Simon D, Guenet J, Gruss P. 1988. A mouse gene homologous to the Drosophila gene caudal is expressed in epithelial cells from the embryonic intestine. *Genes Dev* **2**: 1647-1654.

Eda A, Osawa H, Satoh K, Yanaka I, Kihira K, Ishino Y, Mutoh H, Sugano K. 2003. Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *J Gastroenterol* **38**: 14-22.

Eda A, Osawa H, Yanaka I, Satoh K, Mutoh H, Kihira K, Sugano K. 2002. Expression of homeobox gene CDX2 precedes that of CDX1 during the progression of intestinal metaplasia. *J Gastroenterol* **37**: 94-100.

Edwards MS, Chadda SD, Zhao Z, Barber BL, Sykes DP. 2012. A systematic review of treatment guidelines for metastatic colorectal cancer. *Colorectal Dis* **14**: e31-47.

Ee HC, Erler T, Bhathal PS, Young GP, James RJ. 1995. Cdx-2 homeodomain protein expression in human and rat colorectal adenoma and carcinoma. *Am J Pathol* **147**: 586-592.

El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N *et al.* 2000. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* **404**: 398-402.

Eulalio A, Behm-Ansmant I, Izaurralde E. 2007. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol* **8**: 9-22.

Evans TC, Hunter CP. 2005. Translational control of maternal RNAs. *WormBook : the online review of C. elegans biology*: 1-11.

Faller G, Dimmler A, Rau T, Spaderna S, Hlubek F, Jung A, Kirchner T, Brabletz T. 2004. Evidence for acid-induced loss of Cdx2 expression in duodenal gastric metaplasia. *J Pathol* **203**: 904-908.

Fang R, Santiago NA, Olds LC, Sibley E. 2000. The homeodomain protein Cdx2 regulates lactase gene promoter activity during enterocyte differentiation. *Gastroenterology* **118**: 115-127.

Fearon ER. 2011. Molecular genetics of colorectal cancer. *Annu Rev Pathol* **6**: 479-507.

Fearon ER, Vogelstein B. 1990. A genetic model for colorectal tumorigenesis. *Cell* **61**: 759-767.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* **127**: 2893-2917.

Filipe MI, Jass JR. 1986. Intestinal metaplasia subtypes and cancer risk. in *Gastric carcinoma*, pp. 87-115. Churchill Livingstone, Edinburgh.

Filipe MI, Muñoz N, Matko I, Kato I, Pompe-Kirn V, Jutersek A, Teuchmann S, Benz M, Prijon T. 1994. Intestinal metaplasia types and the risk of gastric cancer: a cohort study in Slovenia. *Int J Cancer* **57**: 324-329.

Fre S, Pallavi SK, Huyghe M, Laé M, Janssen KP, Robine S, Artavanis-Tsakonas S, Louvard D. 2009. Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. *Proc Natl Acad Sci U S A* **106**: 6309-6314.

Friend K, Campbell ZT, Cooke A, Kroll-Conner P, Wickens MP, Kimble J. 2012. A conserved PUF-Ago-eEF1A complex attenuates translation elongation. *Nat Struct Mol Biol* **19**: 176-183.

Funakoshi S, Kong J, Crissey MA, Dang L, Dang D, Lynch JP. 2010. Intestine-specific transcription factor Cdx2 induces E-cadherin function by enhancing the trafficking of E-cadherin to the cell membrane. *Am J Physiol Gastrointest Liver Physiol* **299**: G1054-1067.

Furumiya M, Inoue K, Ohta K, Hayashi Y, Yuasa H. 2013. Transcriptional regulation of PCFT by KLF4, HNF4α, CDX2 and C/EBPα: implication in its site-specific expression in the small intestine. *Biochem Biophys Res Commun* **431**: 158-163.

Gamer LW, Wright CV. 1993. Murine Cdx-4 bears striking similarities to the Drosophila caudal gene in its homeodomain sequence and early expression pattern. *Mech Dev* **43**: 71-81.

Gao N, Kaestner KH. 2010. Cdx2 regulates endo-lysosomal function and epithelial cell polarity. *Genes Dev* **24**: 1295-1305.

Gao N, White P, Kaestner KH. 2009. Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. *Dev Cell* **16**: 588-599.

Gautier-Stein A, Domon-Dell C, Calon A, Bady I, Freund JN, Mithieux G, Rajas F. 2003. Differential regulation of the glucose-6-phosphatase TATA box by intestine-specific homeodomain proteins CDX1 and CDX2. *Nucleic Acids Res* **31**: 5238-5246.

Gebauer F, Hentze MW. 2004. Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol* **5**: 827-835.

Gerber AP, Herschlag D, Brown PO. 2004. Extensive association of functionally and cytotopically related mRNAs with Puf family RNA-binding proteins in yeast. *PLoS Biol* **2**: E79.

Ghosh M, Aguila HL, Michaud J, Ai Y, Wu MT, Hemmes A, Ristimaki A, Guo C, Furneaux H, Hla T. 2009. Essential role of the RNA-binding protein HuR in progenitor cell survival in mice. *J Clin Invest* **119**: 3530-3543.

Glazer RI, Vo DT, Penalva LO. 2012. Musashi1: an RBP with versatile functions in normal and cancer stem cells. *Front Biosci* **17**: 54-64.

Goldenring JR, Nam KT, Mills JC. 2011. The origin of pre-neoplastic metaplasia in the stomach: chief cells emerge from the Mist. *Exp Cell Res* **317**: 2759-2764.

Goldstein B, Macara IG. 2007. The PAR proteins: fundamental players in animal cell polarization. *Dev Cell* **13**: 609-622.

González CA, Sanz-Anquela JM, Gisbert JP, Correa P. 2013. Utility of subtyping intestinal metaplasia as marker of gastric cancer risk. A review of the evidence. *Int J Cancer* **133**: 1023-1032.

Grady WM, Carethers JM. 2008. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* **135**: 1079-1099.

Grainger S, Savory JG, Lohnes D. 2010. Cdx2 regulates patterning of the intestinal epithelium. *Dev Biol* **339**: 155-165.

Groisman GM, Amar M, Meir A. 2004. Expression of the intestinal marker Cdx2 in the columnar-lined esophagus with and without intestinal (Barrett's) metaplasia. *Mod Pathol* **17**: 1282-1288.

Gross I, Duluc I, Benameur T, Calon A, Martin E, Brabletz T, Kedinger M, Domon-Dell C, Freund JN. 2008. The intestine-specific homeobox gene Cdx2 decreases

mobility and antagonizes dissemination of colon cancer cells. *Oncogene* **27**: 107-115.

Gross I, Lhermitte B, Domon-Dell C, Duluc I, Martin E, Gaiddon C, Kedinger M, Freund JN. 2005. Phosphorylation of the homeotic tumor suppressor Cdx2 mediates its ubiquitin-dependent proteasome degradation. *Oncogene* **24**: 7955-7963.

Grover PK, Hardingham JE, Cummins AG. 2010. Stem cell marker olfactomedin 4: critical appraisal of its characteristics and role in tumorigenesis. *Cancer Metastasis Rev* **29**: 761-775.

Guggenheim DE, Shah MA. 2013. Gastric cancer epidemiology and risk factors. *J Surg Oncol* **107**: 230-236.

Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. 1998. E-cadherin germline mutations in familial gastric cancer. *Nature* **392**: 402-405.

Guo M, House MG, Suzuki H, Ye Y, Brock MV, Lu F, Liu Z, Rustgi AK, Herman JG. 2007. Epigenetic silencing of CDX2 is a feature of squamous esophageal cancer. *Int J Cancer* **121**: 1219-1226.

Guo RJ, Funakoshi S, Lee HH, Kong J, Lynch JP. 2010. The intestine-specific transcription factor Cdx2 inhibits beta-catenin/TCF transcriptional activity by disrupting the beta-catenin-TCF protein complex. *Carcinogenesis* **31**: 159-166.

Hall PA, Coates PJ, Ansari B, Hopwood D. 1994. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Sci* **107**: 3569-3577.

Hinkel I, Duluc I, Martin E, Guenot D, Freund JN, Gross I. 2012. Cdx2 controls expression of the protocadherin Mucdhl, an inhibitor of growth and β-catenin activity in colon cancer cells. *Gastroenterology* **142**: 875-885.

Hinoi T, Loda M, Fearon ER. 2003. Silencing of CDX2 expression in colon cancer via a dominant repression pathway. *J Biol Chem* **278**: 44608-44616.

Hinoi T, Lucas PC, Kuick R, Hanash S, Cho KR, Fearon ER. 2002. CDX2 regulates liver intestine-cadherin expression in normal and malignant colon epithelium and intestinal metaplasia. *Gastroenterology* **123**: 1565-1577.

Hinoi T, Tani M, Lucas PC, Caca K, Dunn RL, Macri E, Loda M, Appelman HD, Cho KR, Fearon ER. 2001. Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. *Am J Pathol* **159**: 2239-2248.

Höcker M, Wiedenmann B. 1998. Molecular mechanisms of enteroendocrine differentiation. *Ann N Y Acad Sci* **859**: 160-174.

Hogan DJ, Riordan DP, Gerber AP, Herschlag D, Brown PO. 2008. Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. *PLoS Biol* **6**: e255.

Houde M, Laprise P, Jean D, Blais M, Asselin C, Rivard N. 2001. Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox transcription factor CDX2. *J Biol Chem* **276**: 21885-21894.

Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR, Wang TC. 2004. Gastric cancer originating from bone marrow-derived cells. *Science* **306**: 1568-1571.

Hryniuk A, Grainger S, Savory JG, Lohnes D. 2012. Cdx function is required for maintenance of intestinal identity in the adult. *Dev Biol* **363**: 426-437.

Huang EH, Wicha MS. 2008. Colon cancer stem cells: implications for prevention and therapy. *Trends Mol Med* **14**: 503-509.

Huang NN, Mootz DE, Walhout AJ, Vidal M, Hunter CP. 2002. MEX-3 interacting proteins link cell polarity to asymmetric gene expression in Caenorhabditis elegans. *Development* **129**: 747-759.

Hunter CP, Kenyon C. 1996. Spatial and temporal controls target pal-1 blastomere-specification activity to a single blastomere lineage in C. elegans embryos. *Cell* **87**: 217-226.

Huntzinger E, Izaurralde E. 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* **12**: 99-110.

Huo X, Zhang HY, Zhang XI, Lynch JP, Strauch ED, Wang JY, Melton SD, Genta RM, Wang DH, Spechler SJ *et al.* 2010. Acid and bile salt-induced CDX2 expression differs in esophageal squamous cells from patients with and without Barrett's esophagus. *Gastroenterology* **139**: 194-203.

IARC. 1994. *Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans.*, Lyon.

IARC. 2010. The Globocan Project 2008 (online), globocan.iarc.fr.

Inada K, Tanaka H, Nakanishi H, Tsukamoto T, Ikehara Y, Tatematsu K, Nakamura S, Porter EM, Tatematsu M. 2001. Identification of Paneth cells in pyloric glands associated with gastric and intestinal mixed-type intestinal metaplasia of the human stomach. *Virchows Arch* **439**: 14-20.

Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M *et al.* 2009. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet* **41**: 178-186.

Isshiki S, Kudo T, Nishihara S, Ikehara Y, Togayachi A, Furuya A, Shitara K, Kubota T, Watanabe M, Kitajima M *et al.* 2003. Lewis type 1 antigen synthase (beta3Gal-T5) is transcriptionally regulated by homeoproteins. *J Biol Chem* **278**: 36611-36620.

Jackson RJ, Hellen CU, Pestova TV. 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* **11**: 113-127.

James R, Erler T, Kazenwadel J. 1994. Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. *J Biol Chem* **269**: 15229-15237.

James R, Kazenwadel J. 1991. Homeobox gene expression in the intestinal epithelium of adult mice. *J Biol Chem* **266**: 3246-3251.

Jedrusik A, Bruce AW, Tan MH, Leong DE, Skamagki M, Yao M, Zernicka-Goetz M. 2010. Maternally and zygotically provided Cdx2 have novel and critical roles for early development of the mouse embryo. *Dev Biol* **344**: 66-78.

Jedrusik A, Parfitt DE, Guo G, Skamagki M, Grabarek JB, Johnson MH, Robson P, Zernicka-Goetz M. 2008. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. *Genes Dev* **22**: 2692-2706.

Jiang H, Zhang X, Luo J, Dong C, Xue J, Wei W, Chen J, Zhou J, Gao Y, Yang C. 2012. Knockdown of hMex-3A by small RNA interference suppresses cell proliferation and migration in human gastric cancer cells. *Mol Med Rep* **6**: 575-580.

Jin T, Drucker DJ. 1996. Activation of proglucagon gene transcription through a novel promoter element by the caudal-related homeodomain protein cdx-2/3. *Mol Cell Biol* **16**: 19-28.

Johnson CM, Wei C, Ensor JE, Smolenski DJ, Amos CI, Levin B, Berry DA. 2013. Meta-analyses of colorectal cancer risk factors. *Cancer Causes Control* **24**: 1207-1222.

Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD. 2003. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* **302**: 2141-2144.

Jud M, Razelun J, Bickel J, Czerwinski M, Schisa JA. 2007. Conservation of large foci formation in arrested oocytes of Caenorhabditis nematodes. *Dev Genes Evol* **217**: 221-226.

Kahyo T, Iwaizumi M, Shinmura K, Matsuura S, Nakamura T, Watanabe Y, Yamada H, Sugimura H. 2011. A novel tumor-derived SGOL1 variant causes abnormal mitosis and unstable chromatid cohesion. *Oncogene* **30**: 4453-4463.

Kaimaktchiev V, Terracciano L, Tornillo L, Spichtin H, Stoios D, Bundi M, Korcheva V, Mirlacher M, Loda M, Sauter G *et al.* 2004. The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas. *Mod Pathol* **17**: 1392-1399.

Kakizaki F, Aoki K, Miyoshi H, Carrasco N, Aoki M, Taketo MM. 2010. CDX transcription factors positively regulate expression of solute carrier family 5, member 8 in the colonic epithelium. *Gastroenterology* **138**: 627-635.

Karam SM. 1999. Lineage commitment and maturation of epithelial cells in the gut. *Front Biosci* **4**: D286-298.

Kawai H, Tomii K, Toyooka S, Yano M, Murakami M, Tsukuda K, Shimizu N. 2005. Promoter methylation downregulates CDX2 expression in colorectal carcinomas. *Oncol Rep* **13**: 547-551.

Kazumori H, Ishihara S, Rumi M, Kadowaki Y, Kinoshita Y. 2006. Bile acids directly augment caudal related homeobox gene Cdx2 expression in oesophageal keratinocytes in Barrett's epithelium. *Gut* **55**: 16-25.

Keene JD. 2007. RNA regulons: coordination of post-transcriptional events. *Nat Rev Genet* **8**: 533-543.

Keene JD, Tenenbaum SA. 2002. Eukaryotic mRNPs may represent posttranscriptional operons. *Mol Cell* **9**: 1161-1167.

Kemphues KJ, Priess JR, Morton DG, Cheng NS. 1988. Identification of genes required for cytoplasmic localization in early C. elegans embryos. *Cell* **52**: 311-320.

Kim BM, Buchner G, Miletich I, Sharpe PT, Shivdasani RA. 2005. The stomach mesenchymal transcription factor Barx1 specifies gastric epithelial identity through inhibition of transient Wnt signaling. *Dev Cell* **8**: 611-622.

Kim HS, Lee JS, Freund JN, Min KW, Lee JS, Kim W, Juhng SW, Park CS. 2006. CDX-2 homeobox gene expression in human gastric carcinoma and precursor lesions. *J Gastroenterol Hepatol* **21**: 438-442.

Kim S, Domon-Dell C, Wang Q, Chung DH, Di Cristofano A, Pandolfi PP, Freund JN, Evers BM. 2002. PTEN and TNF-alpha regulation of the intestinal-specific Cdx-2 homeobox gene through a PI3K, PKB/Akt, and NF-kappaB-dependent pathway. *Gastroenterology* **123**: 1163-1178.

Kirchner T, Müller S, Hattori T, Mukaisyo K, Papadopoulos T, Brabletz T, Jung A. 2001. Metaplasia, intraepithelial neoplasia and early cancer of the stomach are related to dedifferentiated epithelial cells defined by cytokeratin-7 expression in gastritis. *Virchows Arch* **439**: 512-522.

Köhler A, Hurt E. 2007. Exporting RNA from the nucleus to the cytoplasm. *Nat Rev Mol Cell Biol* **8**: 761-773.

Krueger F, Madeja Z, Hemberger M, McMahon M, Cook SJ, Gaunt SJ. 2009. Down-regulation of Cdx2 in colorectal carcinoma cells by the Raf-MEK-ERK 1/2 pathway. *Cell Signal* **21**: 1846-1856.

Kuersten S, Goodwin EB. 2003. The power of the 3′ UTR: translational control and development. *Nat Rev Genet* **4**: 626-637.

Lambert M, Colnot S, Suh E, L'Horset F, Blin C, Calliot ME, Raymondjean M, Thomasset M, Traber PG, Perret C. 1996. cis-Acting elements and transcription factors involved in the intestinal specific expression of the rat calbindin-D9K gene:

binding of the intestine-specific transcription factor Cdx-2 to the TATA box. *Eur J Biochem* **236**: 778-788.

Lauren P. 1965. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* **64**: 31-49.

Lee MH, Schedl T. 2001. Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for C. elegans germ cell development. *Genes Dev* **15**: 2408-2420.

Lemieux E, Boucher MJ, Mongrain S, Boudreau F, Asselin C, Rivard N. 2011. Constitutive activation of the MEK/ERK pathway inhibits intestinal epithelial cell differentiation. *Am J Physiol Gastrointest Liver Physiol* **301**: G719-730.

Lengerke C, Daley GQ. 2012. Caudal genes in blood development and leukemia. *Ann N Y Acad Sci* **1266**: 47-54.

Lewis JD, Izaurralde E. 1997. The role of the cap structure in RNA processing and nuclear export. *Eur J Biochem* **247**: 461-469.

Liu Q, Teh M, Ito K, Shah N, Ito Y, Yeoh KG. 2007a. CDX2 expression is progressively decreased in human gastric intestinal metaplasia, dysplasia and cancer. *Mod Pathol* **20**: 1286-1297.

Liu T, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS *et al.* 2007b. Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. *Carcinogenesis* **28**: 488-496.

Lorentz O, Cadoret A, Duluc I, Capeau J, Gespach C, Cherqui G, Freund JN. 1999. Downregulation of the colon tumour-suppressor homeobox gene Cdx-2 by oncogenic ras. *Oncogene* **18**: 87-92.

Lorentz O, Duluc I, Arcangelis AD, Simon-Assmann P, Kedinger M, Freund JN. 1997. Key role of the Cdx2 homeobox gene in extracellular matrix-mediated intestinal cell differentiation. *J Cell Biol* **139**: 1553-1565.

Lu X, Freund JN, Muller M, Ravey J, Nicolas JP, Gueant JL, Namour F. 2008. Differential regulation of CDX1 and CDX2 gene expression by deficiency in methyl group donors. *Biochimie* **90**: 697-704.

Lukong KE, Chang KW, Khandjian EW, Richard S. 2008. RNA-binding proteins in human genetic disease. *Trends Genet* **24**: 416-425.

Lukong KE, Larocque D, Tyner A, Richard S. 2005. Tyrosine phosphorylation of sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression. *J Biol Chem* **280**: 38639-38647.

Lunde BM, Moore C, Varani G. 2007. RNA-binding proteins: modular design for efficient function. *Nat Rev Mol Cell Biol* **8**: 479-490.

Luo Y, Shan G, Guo W, Smrt RD, Johnson EB, Li X, Pfeiffer RL, Szulwach KE, Duan R, Barkho BZ *et al.* 2010. Fragile x mental retardation protein regulates proliferation and differentiation of adult neural stem/progenitor cells. *PLoS Genet* **6**: e1000898.

Mahatan CS, Kaestner KH, Geiman DE, Yang VW. 1999. Characterization of the structure and regulation of the murine gene encoding gut-enriched Krüppel-like factor (Krüppel-like factor 4). *Nucleic Acids Res* **27**: 4562-4569.

Maier T, Güell M, Serrano L. 2009. Correlation of mRNA and protein in complex biological samples. *FEBS Lett* **583**: 3966-3973.

Mallo GV, Rechreche H, Frigerio JM, Rocha D, Zweibaum A, Lacasa M, Jordan BR, Dusetti NJ, Dagorn JC, Iovanna JL. 1997. Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Down-regulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis. *Int J Cancer* **74**: 35-44.

Mallo GV, Soubeyran P, Lissitzky JC, André F, Farnarier C, Marvaldi J, Dagorn JC, Iovanna JL. 1998. Expression of the Cdx1 and Cdx2 homeotic genes leads to reduced malignancy in colon cancer-derived cells. *J Biol Chem* **273**: 14030-14036.

Mangus DA, Evans MC, Jacobson A. 2003. Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. *Genome Biol* **4**: 223.

Marchetti M, Caliot E, Pringault E. 2003. Chronic acid exposure leads to activation of the cdx2 intestinal homeobox gene in a long-term culture of mouse esophageal keratinocytes. *J Cell Sci* **116**: 1429-1436.

Margalit Y, Yarus S, Shapira E, Gruenbaum Y, Fainsod A. 1993. Isolation and characterization of target sequences of the chicken CdxA homeobox gene. *Nucleic Acids Res* **21**: 4915-4922.

Matlin AJ, Clark F, Smith CW. 2005. Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol* **6**: 386-398.

Matsuda K, Yamauchi K, Matsumoto T, Sano K, Yamaoka Y, Ota H. 2008. Quantitative analysis of the effect of Helicobacter pylori on the expressions of SOX2, CDX2, MUC2, MUC5AC, MUC6, TFF1, TFF2, and TFF3 mRNAs in human gastric carcinoma cells. *Scand J Gastroenterol* **43**: 25-33.

Merlos-Suárez A, Barriga FM, Jung P, Iglesias M, Céspedes MV, Rossell D, Sevillano M, Hernando-Momblona X, da Silva-Diz V, Muñoz P *et al.* 2011. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. *Cell Stem Cell* **8**: 511-524.

Mesquita P, Jonckheere N, Almeida R, Ducourouble MP, Serpa J, Silva E, Pigny P, Silva FS, Reis C, Silberg D *et al.* 2003. Human MUC2 mucin gene is

transcriptionally regulated by Cdx homeodomain proteins in gastrointestinal carcinoma cell lines. *J Biol Chem* **278**: 51549-51556.

Mesquita P, Raquel A, Nuno L, Reis CA, Silva LF, Serpa J, Van Seuningen I, Barros H, David L. 2006. Metaplasia--a transdifferentiation process that facilitates cancer development: the model of gastric intestinal metaplasia. *Crit Rev Oncog* **12**: 3-26.

Milano F, van Baal JW, Buttar NS, Rygiel AM, de Kort F, DeMars CJ, Rosmolen WD, Bergman JJ, Van Marle J, Wang KK *et al.* 2007. Bone morphogenetic protein 4 expressed in esophagitis induces a columnar phenotype in esophageal squamous cells. *Gastroenterology* **132**: 2412-2421.

Mizoshita T, Inada K-i, Tsukamoto T, Nozaki K, Joh T, Itoh M, Yamamura Y, Ushijima T, Nakamura S, Tatematsu M. 2004. Expression of the intestine-specific transcription factors, Cdx1 and Cdx2, correlates shift to an intestinal phenotype in gastric cancer cells. *J Cancer Res Clin Oncol* **130**: 29-36.

Mlodzik M, Fjose A, Gehring WJ. 1985. Isolation of caudal, a Drosophila homeo box-containing gene with maternal expression, whose transcripts form a concentration gradient at the pre-blastoderm stage. *EMBO J* **4**: 2961-2969.

Modica S, Morgano A, Salvatore L, Petruzzelli M, Vanier MT, Valanzano R, Esposito DL, Palasciano G, Duluc I, Freund JN *et al.* 2009. Expression and localisation of insulin receptor substrate 2 in normal intestine and colorectal tumours. Regulation by intestine-specific transcription factor CDX2. *Gut* **58**: 1250-1259.

Moore MJ. 2005. From Birth to Death: The Complex Lives of Eukaryotic mRNAs. *Science* **309**: 1514-1518.

Mootz D, Ho DM, Hunter CP. 2004. The STAR/Maxi-KH domain protein GLD-1 mediates a developmental switch in the translational control of C. elegans PAL-1. *Development* **131**: 3263-3272.

Moskaluk CA, Zhang H, Powell SM, Cerilli LA, Hampton GM, Frierson HFJr. 2003. Cdx2 protein expression in normal and malignant human tissues: an immunohistochemical survey using tissue microarrays. *Mod Pathol* **16**: 913-919.

Mutoh H, Hakamata Y, Sato K, Eda A, Yanaka I, Honda S, Osawa H, Kaneko Y, Sugano K. 2002. Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice. *Biochem Biophys Res Commun* **294**: 470-479.

Mutoh H, Hayakawa H, Sakamoto H, Sashikawa M, Sugano K. 2009. Transgenic Cdx2 induces endogenous Cdx1 in intestinal metaplasia of Cdx2-transgenic mouse stomach. *FEBS J* **276**: 5821-5831.

Mutoh H, Hayakawa H, Sashikawa M, Sakamoto H, Sugano K. 2010. Direct repression of Sonic Hedgehog expression in the stomach by Cdx2 leads to intestinal transformation. *Biochem J* **427**: 423-434.

Mutoh H, Sakurai S, Satoh K, Osawa H, Tomiyama T, Kita H, Yoshida T, Tamada K, Yamamoto H, Isoda N *et al.* 2005a. Pericryptal fibroblast sheath in intestinal metaplasia and gastric carcinoma. *Gut* **54**: 33-39.

Mutoh H, Sakurai S, Satoh K, Tamada K, Kita H, Osawa H, Tomiyama T, Sato Y, Yamamoto H, Isoda N *et al.* 2004. Development of gastric carcinoma from intestinal metaplasia in Cdx2-transgenic mice. *Cancer Res* **64**: 7740-7747.

Mutoh H, Satoh K, Kita H, Sakamoto H, Hayakawa H, Yamamoto H, Isoda N, Tamada K, Ido K, Sugano K. 2005b. Cdx2 specifies the differentiation of morphological as well as functional absorptive enterocytes of the small intestine. *Int J Dev Biol* **49**: 867-871.

Nagaoka K, Udagawa T, Richter JD. 2012. CPEB-mediated ZO-1 mRNA localization is required for epithelial tight-junction assembly and cell polarity. *Nat Commun* **3**: 675.

Niessing D, Blanke S, Jäckle H. 2002. Bicoid associates with the 5'-cap-bound complex of caudal mRNA and represses translation. *Genes Dev* **16**: 2576-2582.

Nishioka N, Inoue K-i, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N *et al.* 2009. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Dev Cell* **16**: 398-410.

Niwa T, Ikehara Y, Nakanishi H, Tanaka H, Inada K-i, Tsukamoto T, Ichinose M, Tatematsu M. 2005. Mixed gastric- and intestinal-type metaplasia is formed by cells with dual intestinal and gastric differentiation. *J Histochem Cytochem* **53**: 75-85.

Olsen AK, Coskun M, Bzorek M, Kristensen MH, Danielsen ET, Jørgensen S, Olsen J, Engel U, Holck S, Troelsen JT. 2013. Regulation of APC and AXIN2 expression by intestinal tumor suppressor CDX2 in colon cancer cells. *Carcinogenesis* **34**: 1361-1369.

Ostareck DH, Ostareck-Lederer A, Shatsky IN, Hentze MW. 2001. Lipoxygenase mRNA silencing in erythroid differentiation: The 3'UTR regulatory complex controls 60S ribosomal subunit joining. *Cell* **104**: 281-290.

Pacheco II, Macleod RJ. 2008. CaSR stimulates secretion of Wnt5a from colonic myofibroblasts to stimulate CDX2 and sucrase-isomaltase using Ror2 on intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* **295**: G748-759.

Pagano JM, Farley BM, Essien KI, Ryder SP. 2009. RNA recognition by the embryonic cell fate determinant and germline totipotency factor MEX-3. *Proc Natl Acad Sci U S A* **106**: 20252-20257.

Pallafacchina G, François S, Regnault B, Czarny B, Dive V, Cumano A, Montarras D, Buckingham M. 2010. An adult tissue-specific stem cell in its niche: a gene profiling analysis of in vivo quiescent and activated muscle satellite cells. *Stem Cell Res* **4**: 77-91.

Palles C, Cazier JB, Howarth KM, Domingo E, Jones AM, Broderick P, Kemp Z, Spain SL, Guarino E, Guarino Almeida E *et al.* 2013. Germline mutations affecting the proofreading domains of POLE and POLD1 predispose to colorectal adenomas and carcinomas. *Nat Genet* **45**: 136-144.

Park J, Schulz S, Waldman SA. 2000. Intestine-specific activity of the human guanylyl cyclase C promoter is regulated by Cdx2. *Gastroenterology* **119**: 89-96.

Porter EM, Bevins CL, Ghosh D, Ganz T. 2002. The multifaceted Paneth cell. *Cell Mol Life Sci* **59**: 156-170.

Potten CS. 1977. Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation. *Nature* **269**: 518-521.

Potten CS, Booth C, Tudor GL, Booth D, Brady G, Hurley P, Ashton G, Clarke R, Sakakibara S-i, Okano H. 2003. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. *Differentiation* **71**: 28-41.

Potten CS, Kovacs L, Hamilton E. 1974. Continuous labelling studies on mouse skin and intestine. *Cell Tissue Kinet* **7**: 271-283.

Potten CS, Loeffler M. 1990. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110**: 1001-1020.

Potten CS, Owen G, Booth D. 2002. Intestinal stem cells protect their genome by selective segregation of template DNA strands. *J Cell Sci* **115**: 2381-2388.

Pritchard CC, Grady WM. 2011. Colorectal cancer molecular biology moves into clinical practice. *Gut* **60**: 116-129.

Ralston A, Rossant J. 2008. Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo. *Dev Biol* **313**: 614-629.

Reis CA, David L, Correa P, Carneiro F, de Bolós C, Garcia E, Mandel U, Clausen H, Sobrinho-Simões M. 1999. Intestinal metaplasia of human stomach displays distinct patterns of mucin (MUC1, MUC2, MUC5AC, and MUC6) expression. *Cancer Res* **59**: 1003-1007.

Rhoads RE. 2009. eIF4E: new family members, new binding partners, new roles. *J Biol Chem* **284**: 16711-16715.

Rings EH, Boudreau F, Taylor JK, Moffett J, Suh ER, Traber PG. 2001. Phosphorylation of the serine 60 residue within the Cdx2 activation domain mediates its transactivation capacity. *Gastroenterology* **121**: 1437-1450.

Rokkas T, Filipe MI, Sladen GE. 1991. Detection of an increased incidence of early gastric cancer in patients with intestinal metaplasia type III who are closely followed up. *Gut* **32**: 1110-1113.

Rotkrua P, Akiyama Y, Hashimoto Y, Otsubo T, Yuasa Y. 2011. MiR-9 downregulates CDX2 expression in gastric cancer cells. *Int J Cancer* **129**: 2611-2620.

Sakagami Y, Inaguma Y, Sakakura T, Nishizuka Y. 1984. Intestine-like remodeling of adult mouse glandular stomach by implanting of fetal intestinal mesenchyme. *Cancer Res* **44**: 5845-5849.

Sakaguchi T, Gu X, Golden HM, Suh E, Rhoads DB, Reinecker HC. 2002. Cloning of the human claudin-2 5'-flanking region revealed a TATA-less promoter with conserved binding sites in mouse and human for caudal-related homeodomain proteins and hepatocyte nuclear factor-1alpha. *J Biol Chem* **277**: 21361-21370.

Sakamoto H, Mutoh H, Hayakawa H, Sashikawa M, Sugano K. 2011. Cell lineage dynamics in the process leading to intestinal metaplasia. *J Gastroenterol* **46**: 620-628.

Sakamoto H, Mutoh H, Ido K, Satoh K, Hayakawa H, Sugano K. 2007. A close relationship between intestinal metaplasia and Cdx2 expression in human gallbladders with cholelithiasis. *Hum Pathol* **38**: 66-71.

Salari K, Spulak ME, Cuff J, Forster AD, Giacomini CP, Huang S, Ko ME, Lin AY, van de Rijn M, Pollack JR. 2012. CDX2 is an amplified lineage-survival oncogene in colorectal cancer. *Proc Natl Acad Sci U S A* **109**: E3196-3205.

Sampath P, Pritchard DK, Pabon L, Reinecke H, Schwartz SM, Morris DR, Murry CE. 2008. A hierarchical network controls protein translation during murine embryonic stem cell self-renewal and differentiation. *Cell Stem Cell* **2**: 448-460.

Sangiorgi E, Capecchi MR. 2008. Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* **40**: 915-920.

Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ *et al.* 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**: 262-265.

Satoh K, Mutoh H, Eda A, Yanaka I, Osawa H, Honda S, Kawata H, Kihira K, Sugano K. 2002. Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of Helicobacter pylori. *Helicobacter* **7**: 192-198.

Satou Y. 1999. posterior end mark 3 (pem-3), an ascidian maternally expressed gene with localized mRNA encodes a protein with Caenorhabditis elegans MEX-3-like KH domains. *Dev Biol* **212**: 337-350.

Savory JG, Pilon N, Grainger S, Sylvestre JR, Béland M, Houle M, Oh K, Lohnes D. 2009. Cdx1 and Cdx2 are functionally equivalent in vertebral patterning. *Dev Biol* **330**: 114-122.

Schisa JA, Pitt JN, Priess JR. 2001. Analysis of RNA associated with P granules in germ cells of C. elegans adults. *Development* **128**: 1287-1298.

Schonhoff SE, Giel-Moloney M, Leiter AB. 2004. Minireview: Development and differentiation of gut endocrine cells. *Endocrinology* **145**: 2639-2644.

Schoppmeier M, Fischer S, Schmitt-Engel C, Löhr U, Klingler M. 2009. An ancient anterior patterning system promotes caudal repression and head formation in ecdysozoa. *Curr Biol* **19**: 1811-1815.

Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M. 2011. Global quantification of mammalian gene expression control. *Nature* **473**: 337-342.

Selgrad M, Bornschein J, Rokkas T, Malfertheiner P. 2010. Clinical aspects of gastric cancer and Helicobacter pylori--screening, prevention, and treatment. *Helicobacter* **15**: 40-45.

Semba S, Satake S, Matsushita M, Yokozaki H. 2009. Phosphatase activity of nuclear PTEN is required for CDX2-mediated intestinal differentiation of gastric carcinoma. *Cancer Lett* **274**: 143-150.

Seydoux G, Fire A. 1994. Soma-germline asymmetry in the distributions of embryonic RNAs in Caenorhabditis elegans. *Development* **120**: 2823-2834.

Shimada T, Koike T, Yamagata M, Yoneda M, Hiraishi H. 2007. Regulation of TFF3 expression by homeodomain protein CDX2. *Regul Pept* **140**: 81-87.

Silberg DG, Furth EE, Taylor JK, Schuck T, Chiou T, Traber PG. 1997. CDX1 protein expression in normal, metaplastic, and neoplastic human alimentary tract epithelium. *Gastroenterology* **113**: 478-486.

Silberg DG, Sullivan J, Kang E, Swain GP, Moffett J, Sund NJ, Sackett SD, Kaestner KH. 2002. Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. *Gastroenterology* **122**: 689-696.

Silberg DG, Swain GP, Suh ER, Traber PG. 2000. Cdx1 and cdx2 expression during intestinal development. *Gastroenterology* **119**: 961-971.

Silva E, Teixeira A, David L, Carneiro F, Reis CA, Sobrinho-Simões J, Serpa J, Veerman E, Bolscher J, Sobrinho-Simões M. 2002. Mucins as key molecules for the classification of intestinal metaplasia of the stomach. *Virchows Arch* **440**: 311-317.

Siomi H, Matunis MJ, Michael WM, Dreyfuss G. 1993. The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. *Nucleic Acids Res* **21**: 1193-1198.

Skamagki M, Wicher KB, Jedrusik A, Ganguly S, Zernicka-Goetz M. 2013. Asymmetric localization of Cdx2 mRNA during the first cell-fate decision in early mouse development. *Cell Rep* **3**: 442-457.

Slack JM. 2007. Metaplasia and transdifferentiation: from pure biology to the clinic. *Nat Rev Mol Cell Biol* **8**: 369-378.

Stebbins-Boaz B, Cao Q, de Moor CH, Mendez R, Richter JD. 1999. Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. *Mol Cell* **4**: 1017-1027.

Stringer EJ, Duluc I, Saandi T, Davidson I, Bialecka M, Sato T, Barker N, Clevers H, Pritchard CA, Winton DJ *et al.* 2012. Cdx2 determines the fate of postnatal intestinal endoderm. *Development* **139**: 465-474.

Stringer EJ, Pritchard CA, Beck F. 2008. Cdx2 initiates histodifferentiation of the midgut endoderm. *FEBS Lett* **582**: 2555-2560.

Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J. 2005. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* **132**: 2093-2102.

Subramanian V, Meyer BI, Gruss P. 1995. Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell* **83**: 641-653.

Subtil C, Guérin E, Schneider A, Chenard MP, Martin E, Domon-Dell C, Duluc I, Brabletz T, Kedinger M, Duclos B *et al.* 2007. Frequent rearrangements and amplification of the CDX2 homeobox gene in human sporadic colorectal cancers with chromosomal instability. *Cancer Lett* **247**: 197-203.

Suh E, Chen L, Taylor J, Traber PG. 1994. A homeodomain protein related to caudal regulates intestine-specific gene transcription. *Mol Cell Biol* **14**: 7340-7351.

Suh E, Traber PG. 1996. An intestine-specific homeobox gene regulates proliferation and differentiation. *Mol Cell Biol* **16**: 619-625.

Suvà ML, Riggi N, Bernstein BE. 2013. Epigenetic reprogramming in cancer. *Science* **339**: 1567-1570.

Tagawa T, Haraguchi T, Hiramatsu H, Kobayashi K, Sakurai K, Inada K-I, Iba H. 2012. Multiple microRNAs induced by Cdx1 suppress Cdx2 in human colorectal tumour cells. *Biochem J* **447**: 449-455.

Tamai Y, Nakajima R, Ishikawa T, Takaku K, Seldin MF, Taketo MM. 1999. Colonic hamartoma development by anomalous duplication in Cdx2 knockout mice. *Cancer Res* **59**: 2965-2970.

Taupin D, Podolsky DK. 1999. Mitogen-activated protein kinase activation regulates intestinal epithelial differentiation. *Gastroenterology* **116**: 1072-1080.

Taylor JK, Levy T, Suh ER, Traber PG. 1997. Activation of enhancer elements by the homeobox gene Cdx2 is cell line specific. *Nucleic Acids Res* **25**: 2293-2300.

Tosh D, Slack JM. 2002. How cells change their phenotype. *Nat Rev Mol Cell Biol* **3**: 187-194.

Trinh KY, Jin T, Drucker DJ. 1999. Identification of domains mediating transcriptional activation and cytoplasmic export in the caudal homeobox protein Cdx-3. *J Biol Chem* **274**: 6011-6019.

Troelsen JT, Mitchelmore C, Spodsberg N, Jensen AM, Norén O, Sjöström H. 1997. Regulation of lactase-phlorizin hydrolase gene expression by the caudal-related homoeodomain protein Cdx-2. *Biochem J* **322**: 833-838.

Tsukamoto T, Inada K, Tanaka H, Mizoshita T, Mihara M, Ushijima T, Yamamura Y, Nakamura S, Tatematsu M. 2004. Down-regulation of a gastric transcription factor, Sox2, and ectopic expression of intestinal homeobox genes, Cdx1 and Cdx2: inverse correlation during progression from gastric/intestinal-mixed to complete intestinal metaplasia. *J Cancer Res Clin Oncol* **130**: 135-145.

Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. 2001. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* **345**: 784-789.

Uesaka T, Lu H, Katoh O, Watanabe H. 2002. Heparin-binding EGF-like growth factor gene transcription regulated by Cdx2 in the intestinal epithelium. *Am J Physiol Gastrointest Liver Physiol* **283**: G840-847.

Updike D, Strome S. 2010. P granule assembly and function in Caenorhabditis elegans germ cells. *J Androl* **31**: 53-60.

Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M. 2004. Stem cells and cancer; the polycomb connection. *Cell* **118**: 409-418.

Valverde R, Edwards L, Regan L. 2008. Structure and function of KH domains. *FEBS J* **275**: 2712-2726.

van den Akker E, Forlani S, Chawengsaksophak K, de Graaff W, Beck F, Meyer BI, Deschamps J. 2002. Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* **129**: 2181-2193.

van der Flier LG, Clevers H. 2009. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol* **71**: 241-260.

van der Flier LG, Haegebarth A, Stange DE, van de Wetering M, Clevers H. 2009. OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* **137**: 15-17.

van Nes J, de Graaff W, Lebrin F, Gerhard M, Beck F, Deschamps J. 2006. The Cdx4 mutation affects axial development and reveals an essential role of Cdx genes in the ontogenesis of the placental labyrinth in mice. *Development* **133**: 419-428.

Vaninetti N, Williams L, Geldenhuys L, Porter GA, Guernsey DL, Casson AG. 2009. Regulation of CDX2 expression in esophageal adenocarcinoma. *Mol Carcinog* **48**: 965-974.

Varon C, Dubus P, Mazurier F, Asencio C, Chambonnier L, Ferrand J, Giese A, Senant-Dugot N, Carlotti M, Mégraud F. 2012. Helicobacter pylori infection recruits

bone marrow-derived cells that participate in gastric preneoplasia in mice. *Gastroenterology* **142**: 281-291.

Vauhkonen M, Vauhkonen H, Sipponen P. 2008. Helicobacter pylori infection induces a reversible expression of the CDX2 transcription factor protein in human gastric epithelium. *Scand J Gastroenterol* **43**: 915-921.

Verzi MP, Shin H, He HH, Sulahian R, Meyer CA, Montgomery RK, Fleet JC, Brown M, Liu XS, Shivdasani RA. 2010. Differentiation-specific histone modifications reveal dynamic chromatin interactions and partners for the intestinal transcription factor CDX2. *Dev Cell* **19**: 713-726.

Verzi MP, Shin H, Ho LL, Liu XS, Shivdasani RA. 2011. Essential and redundant functions of caudal family proteins in activating adult intestinal genes. *Mol Cell Biol* **31**: 2026-2039.

Verzi MP, Shin H, San Roman AK, Liu X, Shivdasani RA. 2013. Intestinal master transcription factor CDX2 controls chromatin access for partner transcription factor binding. *Mol Cell Biol* **33**: 281-292.

Vogel C, Abreu RS, Ko D, Le SY, Shapiro BA, Burns SC, Sandhu D, Boutz DR, Marcotte EM, Penalva LO. 2010. Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. *Mol Syst Biol* **6**: 400.

Walters JR, Howard A, Rumble HE, Prathalingam SR, Shaw-Smith CJ, Legon S. 1997. Differences in expression of homeobox transcription factors in proximal and distal human small intestine. *Gastroenterology* **113**: 472-477.

Wang L, Klopot A, Freund JN, Dowling LN, Krasinski SD, Fleet JC. 2004a. Control of differentiation-induced calbindin-D9k gene expression in Caco-2 cells by cdx-2 and HNF-1alpha. *Am J Physiol Gastrointest Liver Physiol* **287**: G943-953.

Wang ML, Shin ME, Knight PA, Artis D, Silberg DG, Suh E, Wu GD. 2005. Regulation of RELM/FIZZ isoform expression by Cdx2 in response to innate and adaptive immune stimulation in the intestine. *Am J Physiol Gastrointest Liver Physiol* **288**: G1074-1083.

Wang QT, Piotrowska K, Ciemerych MA, Milenkovic L, Scott MP, Davis RW, Zernicka-Goetz M. 2004b. A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. *Dev Cell* **6**: 133-144.

Wang Y, Yabuuchi A, McKinney-Freeman S, Ducharme DM, Ray MK, Chawengsaksophak K, Archer TK, Daley GQ. 2008. Cdx gene deficiency compromises embryonic hematopoiesis in the mouse. *Proc Natl Acad Sci U S A* **105**: 7756-7761.

Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. 1998. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. *Gastroenterology* **115**: 642-648.

Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* **137**: 231-245.

Wells SE, Hillner PE, Vale RD, Sachs AB. 1998. Circularization of mRNA by eukaryotic translation initiation factors. *Mol Cell* **2**: 135-140.

Werling RW, Yaziji H, Bacchi CE, Gown AM. 2003. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol* **27**: 303-310.

Wicking C, Simms LA, Evans T, Walsh M, Chawengsaksophak K, Beck F, Chenevix-Trench G, Young J, Jass J, Leggett B *et al.* 1998. CDX2, a human homologue of Drosophila caudal, is mutated in both alleles in a replication error positive colorectal cancer. *Oncogene* **17**: 657-659.

Witek ME, Nielsen K, Walters R, Hyslop T, Palazzo J, Schulz S, Waldman SA. 2005. The putative tumor suppressor Cdx2 is overexpressed by human colorectal adenocarcinomas. *Clin Cancer Res* **11**: 8549-8556.

Woodford-Richens KL, Halford S, Rowan A, Bevan S, Aaltonen LA, Wasan H, Bicknell D, Bodmer WF, Houlston RS, Tomlinson IP. 2001. CDX2 mutations do not account for juvenile polyposis or Peutz-Jeghers syndrome and occur infrequently in sporadic colorectal cancers. *Br J Cancer* **84**: 1314-1316.

Wright NA, Irwin M. 1982. The kinetics of villus cell populations in the mouse small intestine. I. Normal villi: the steady state requirement. *Cell Tissue Kinet* **15**: 595-609.

Wu G, Gentile L, Fuchikami T, Sutter J, Psathaki K, Esteves TC, Araúzo-Bravo MJ, Ortmeier C, Verberk G, Abe K *et al.* 2010. Initiation of trophectoderm lineage specification in mouse embryos is independent of Cdx2. *Development* **137**: 4159-4169.

Wu XS, Akiyama Y, Igari T, Kawamura T, Hiranuma S, Shibata T, Tsuruta K, Koike M, Arii S, Yuasa Y. 2005. Expression of homeodomain protein CDX2 in gallbladder carcinomas. *J Cancer Res Clin Oncol* **131**: 271-278.

Xu F, Li H, Jin T. 1999. Cell type-specific autoregulation of the Caudal-related homeobox gene Cdx-2/3. *J Biol Chem* **274**: 34310-34316.

Yamamichi N, Inada K-i, Furukawa C, Sakurai K, Tando T, Ishizaka A, Haraguchi T, Mizutani T, Fujishiro M, Shimomura R *et al.* 2009. Cdx2 and the Brm-type SWI/SNF complex cooperatively regulate villin expression in gastrointestinal cells. *Exp Cell Res* **315**: 1779-1789.

Yamamoto H, Bai YQ, Yuasa Y. 2003. Homeodomain protein CDX2 regulates goblet-specific MUC2 gene expression. *Biochem Biophys Res Commun* **300**: 813-818.

Yamaoka Y. 2010. Mechanisms of disease: Helicobacter pylori virulence factors. *Nat Rev Gastroenterol Hepatol* **7**: 629-641.

Yang G, Fu H, Zhang J, Lu X, Yu F, Jin L, Bai L, Huang B, Shen L, Feng Y *et al.* 2010. RNA-binding protein quaking, a critical regulator of colon epithelial differentiation and a suppressor of colon cancer. *Gastroenterology* **138**: 231-240.

Yang G, Lu X, Wang L, Bian Y, Fu H, Wei M, Pu J, Jin L, Yao L, Lu Z. 2011. E2F1 and RNA binding protein QKI comprise a negative feedback in the cell cycle regulation. *Cell Cycle* **10**: 2703-2713.

Yousef F, Cardwell C, Cantwell MM, Galway K, Johnston BT, Murray L. 2008. The incidence of esophageal cancer and high-grade dysplasia in Barrett's esophagus: a systematic review and meta-analysis. *Am J Epidemiol* **168**: 237-249.

Yuan J, Nguyen CK, Liu X, Kanellopoulou C, Muljo SA. 2012. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science* **335**: 1195-1200.

Yuasa Y, Nagasaki H, Akiyama Y, Hashimoto Y, Takizawa T, Kojima K, Kawano T, Sugihara K, Imai K, Nakachi K. 2009. DNA methylation status is inversely correlated with green tea intake and physical activity in gastric cancer patients. *Int J Cancer* **124**: 2677-2682.

Yuasa Y, Nagasaki H, Akiyama Y, Sakai H, Nakajima T, Ohkura Y, Takizawa T, Koike M, Tani M, Iwai T *et al.* 2005. Relationship between CDX2 gene methylation and dietary factors in gastric cancer patients. *Carcinogenesis* **26**: 193-200.

Zhang J, Roberts TM, Shivdasani RA. 2011. Targeting PI3K signaling as a therapeutic approach for colorectal cancer. *Gastroenterology* **141**: 50-61.

Zhang JF, Zhang JG, Kuai XL, Zhang H, Jiang W, Ding WF, Li ZL, Zhu HJ, Mao ZB. 2013. Reactivation of the homeotic tumor suppressor gene CDX2 by 5-aza-2'-deoxycytidine-induced demethylation inhibits cell proliferation and induces caspase-independent apoptosis in gastric cancer cells. *Exp Ther Med* **5**: 735-741.

Zheng L, Wang L, Ajani J, Xie K. 2004a. Molecular basis of gastric cancer development and progression. *Gastric Cancer* **7**: 61-77.

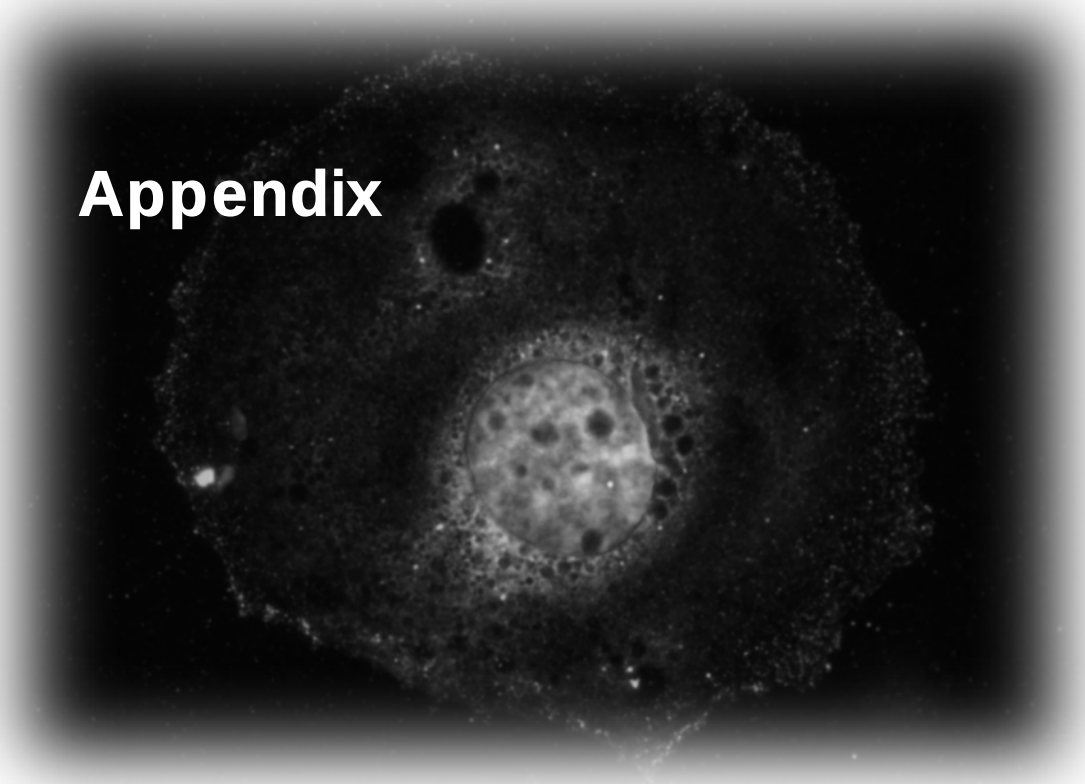
Zheng Q, Chen XY, Shi Y, Xiao SD. 2004b. Development of gastric adenocarcinoma in Mongolian gerbils after long-term infection with Helicobacter pylori. *J Gastroenterol Hepatol* **19**: 1192-1198.

Zhou G, Sun YG, Wang HB, Wang WQ, Wang XW, Fang DC. 2009. Acid and bile salt up-regulate BMP4 expression in human esophageal epithelium cells. *Scand J Gastroenterol* **44**: 926-932.

Zhou VW, Goren A, Bernstein BE. 2011. Charting histone modifications and the functional organization of mammalian genomes. *Nat Rev Genet* **12**: 7-18.

Ziskin JL, Dunlap D, Yaylaoglu M, Fodor IK, Forrest WF, Patel R, Ge N, Hutchins GG, Pine JK, Quirke P *et al.* 2013. In situ validation of an intestinal stem cell signature in colorectal cancer. *Gut* **62**: 1012-1023.

Appendix



Appendix A. List of antibodies and primers used in this study.

ANTIBODIES

Antigen	Application	Description	Dilution	Source	Commercial reference
Actin	WB	Rabbit polyclonal	1:8000	Santa Cruz Biotechnology	sc-1616-R
CDX2	IF and WB	Mouse monoclonal IgG1 (Clone CDX2-88)	1:50 and 1:1000	Biogenex	MU392A-UC
Cyclin D1	WB	Rabbit monoclonal (Clone SP4)	1:500	Thermo Scientific	RM-9104
DCP1A	IF	Rabbit polyclonal	1:500	Sigma Aldrich	D5444
E-cadherin	IF	Mouse monoclonal IgG1 (Clone HECD-1)	1:300	Takara Biochemicals	M106
EDC4	IF	Rabbit polyclonal	1:400	Cell Signaling Technology	2548
F-actin	IF	Tetramethylrhodamine B isothiocyanate (TRITC)-conjugate of phalloidin	1:10000	Sigma Aldrich	77418
MEX3A	IF and WB	Rabbit polyclonal	1:600 and 1:1000	Sigma Aldrich	PRS4869
MSI1	WB	Rabbit polyclonal	1:1000	Millipore	AB5977
c-Myc	IF and WB	Mouse monoclonal IgG1 (Clone 9E10)	1:600 and 1:1000	Clontech	631206
Villin	WB	Mouse monoclonal	1:3000	Kindly provided by Dr. Sylvie Robine, Institute Curie, Paris, France	—
ZO-1	IF	Rabbit polyclonal	1:50	Invitrogen	61-7300
Secondary					
Goat anti-Mouse IgG	IF	Alexa Fluor 488 or Alexa Fluor 594 conjugated	1:100	Invitrogen	A-11001 or A-11005
Goat anti-Mouse IgG	WB	Horseradish peroxidase (HRP) conjugated	1:2000	Santa Cruz Biotechnology	sc-2005
Goat anti-Rabbit IgG	IF	Alexa Fluor 488 or Alexa Fluor 594 conjugated	1:100	Invitrogen	A-11008 or A-11012
Goat anti-Rabbit IgG	WB	Horseradish peroxidase (HRP) conjugated	1:2000	Santa Cruz Biotechnology	sc-2004

PRIMER SEQUENCES (5`- 3`)

Bisulfite sequencing		
	CpG1	F: TTTTAGAAATGATAGGATGAAGG
		R: ACCAAAAAACCTAAAACTAAAAA
	CpG2.1	F: GTAGGTTAGAGGGAGGGAT
		R: TCCTTATCCAAAAAATAACTCAC
	CpG2.2	F: GAGTTATTTTTTGGATAAGGA
		R: TAACCATTCCAATCCTCCC
	CpG2.3	F: GGGAGGATTGGAATGGTTA
		R: TTTACAACAACCCAAAAAC
qPCR		
	18S	F: CGCCGCTAGAGGTGAAATTC
		R: CATTCTTGGCAAATGCTTTCG
	BMI1	F: GTCTACATTCCTTCTGTAAAACG
		R: CTTGGAGAGTTTTATCTGACC
	CDX2	F: TTCACTACAGTCGCTACATCACCAT
		R: TTGTTGATTTTCCTCTCCTTTGCT
	GAPDH	F: TCAAGGCTGAGAACGGGAAG
		R: AGAGGGGGCAGAGATGATGA
	LGR5	F: CAGCGTCTTCACCTCCTACCTA
		R: CCTTGGGAATGTATGTCAGAGC
	MEX3A	F: CAAGCTCTGCGCTCTCTACAAA
		R: GGCCTTAATCTTGCAGCCTTG
	Rluc	F: TGCAAGCAAATGAACGTGCTG
		R: TCTAGCCACGGGCTCGATGT
Site-directed mutagenesis		
	pRLΔCDX2 vector*	S: GTAACATCCAAGCCAG**CC**TTTT**CC**AAGCCTTCTGGATCC
		AS:GGATCCAGAAGGCTT**GG**AAAA**GG**CTGGCTTGGATGTTAC

* Mutated nucleotides are in bold and underlined

Appendix B. List of differentially expressed genes between 3D and 2D AGS cultures.

Gene order	Parametric *P* value	FDR (false discovery rate)	Geom mean of intensities in class 1 (2D)	Geom mean of intensities in class 2 (3D)	Fold-change	Regulation (3D Vs 2D)	Genbank accession	Symbol	Gene name
1	0,000042	0,0207	3691,27	6119,5	1,66	Up	NM_016733	**LIMK2**	LIM domain kinase 2, transcript variant 2b
2	0,000051	0,0207	196,51	37,01	5,31	Down	NM_002135	**NR4A1**	Nuclear receptor subfamily 4, group A, member 1, transcript variant 1
3	0,000053	0,0207	339,43	591,01	1,74	Up	NM_152361	**EID2B**	EP300 interacting inhibitor of differentiation 2B
4	0,000072	0,0207	28,48	44,5	1,56	Up	XM_001716238	**LOC100128081**	Hypothetical LOC100128081
5	0,000084	0,0207	14,16	3,62	3,92	Down	NM_174901	**FAM9C**	Family with sequence similarity 9, member C
6	0,000100	0,0207	5,87	12,49	2,13	Up	NM_001080443	**KIF18B**	Kinesin family member 18B
7	0,000131	0,0207	4,97	49,26	9,91	Up	NM_001146037	**SLC14A1**	Solute carrier family 14 (urea transporter), member 1 (Kidd blood group), transcript variant 4
8	0,000142	0,0207	1414,77	850,58	1,66	Down	NM_022164	**TINAGL1**	Tubulointerstitial nephritis antigen-like 1
9	0,000159	0,0207	26,26	42,57	1,62	Up	NM_152458	**ZNF785**	Zinc finger protein 785
10	0,000181	0,0207	842,04	397,48	2,12	Down	XR_037929	**LOC100130141**	Similar to Kruppel-like factor pseudogene
11	0,000221	0,0207	27518,15	12352,07	2,23	Down	NM_016270	**KLF2**	Kruppel-like factor 2 (lung)
12	0,000249	0,0207	145,56	222,54	1,53	Up	NM_145165	**CHURC1**	Churchill domain containing 1
13	0,000260	0,0207	40,3	92,64	2,30	Up	DW419002	**HHAGE001732**	Human liver regeneration after partial hepatectomy (*H. sapiens*) cDNA
14	0,000296	0,0207	58,8	98,89	1,68	Up	NM_001012410	**SGOL1**	Shugoshin-like 1 (S. pombe), transcript variant A2
15	0,000300	0,0207	74,44	123,58	1,66	Up	NR_002767	**NCRNA00120**	Non-protein coding RNA 120
16	0,000317	0,0207	14,19	28,99	2,04	Up	ENST00000390298	**IGLV7-43**	Immunoglobulin lambda variable 7-43
17	0,000318	0,0207	1554,46	2500,88	1,61	Up	NM_032358	**CCDC77**	Coiled-coil domain containing 77, transcript variant 1
18	0,000324	0,0207	5,89	11,47	1,95	Up	NM_198180	**QRFP**	Pyroglutamylated RFamide peptide
19	0,000329	0,0207	208,27	101,34	2,06	Down	NM_006186	**NR4A2**	Nuclear receptor subfamily 4, group A, member 2
20	0,000355	0,0207	67,66	124,44	1,84	Up	NM_001289	**CLIC2**	Chloride intracellular channel 2
21	0,000365	0,0207	324,74	562,8	1,73	Up	NM_001093725	**MEX3A**	Mex-3 homolog A (*C. elegans*)

22	0,000384	0,0207	983,4	1522,46	1,55	Up	NM_003748	**ALDH4A1**	Aldehyde dehydrogenase 4 family, member A1, nuclear gene encoding mitochondrial protein, transcript variant P5CDhL
23	0,000387	0,0207	356,55	544,92	1,53	Up	NM_017776	**ZNF673**	Zinc finger family member 673, transcript variant 2
24	0,000388	0,0207	262,05	424,56	1,62	Up	NM_017669	**ERCC6L**	Excision repair cross-complementing rodent repair deficiency, complementation group 6-like
25	0,000393	0,0207	39,99	111,17	2,78	Up	NM_005780	**LHFP**	Lipoma HMGIC fusion partner
26	0,000409	0,0207	19,62	3,66	5,36	Down	ENST00000304105	**OR7A2P**	Olfactory receptor, family 7, subfamily A, member 2 pseudogene
27	0,000443	0,0207	31,32	49,79	1,59	Up	NM_053001	**OSR2**	Odd-skipped related 2 (Drosophila), transcript variant 2
28	0,000506	0,0207	125,25	192,61	1,54	Up	NM_018134	**IQCC**	IQ motif containing C, transcript variant 2
29	0,000507	0,0207	23,7	37,83	1,60	Up	ENST00000269201	**C18orf2**	Chromosome 18 open reaing frame 2
30	0,000510	0,0207	7690,13	13468,34	1,75	Up	NM_002691	**POLD1**	Polymerase (DNA directed), delta 1, catalytic subunit 125kDa
31	0,000535	0,0207	25,47	45,56	1,79	Up	ENST00000375520	**C10orf126**	Chromosome 10 open reading frame 126
32	0,000547	0,0207	14,42	4,37	3,30	Down	NM_194463	**RNF128**	Ring finger protein 128, transcript variant 1
33	0,000610	0,0207	7,29	3,87	1,88	Down	NM_001080511	**CLEC2L**	C-type lectin domain family 2, member L
34	0,000622	0,0207	522,86	784,73	1,50	Up	NM_015361	**R3HDM1**	R3H domain containing 1
35	0,000637	0,0207	4,9	12,63	2,58	Up	NM_001011703	**FAM125B**	Family with sequence similarity 125, member B, transcript variant 2
36	0,000654	0,0207	3366,74	5800,5	1,72	Up	XR_042100	**CRNDE**	Colorectal neoplasia differentially expressed (non-protein coding), transcript variant 1
37	0,000806	0,0207	12,3	33,95	2,76	Up	XM_002345453	**LOC100292961**	Hypothetical protein LOC100292961
38	0,000857	0,0207	6,39	3,93	1,63	Down	NM_000044	**AR**	Androgen receptor, transcript variant 1
39	0,000885	0,0207	5277,12	829,16	6,36	Down	NM_002423	**MMP7**	Matrix metallopeptidase 7 (matrilysin, uterine)
40	0,000922	0,0207	55,06	103,67	1,88	Up	BC052561	**STK17**b	Serine/threonine kinase 17b (apoptosis-inducing), mRNA, (cDNA clone IMAGE:6280720), partial cds
41	0,000958	0,0207	17,19	47,95	2,79	Up	XM_001717262	**LOC646509**	Hypothetical protein LOC646509
42	0,000963	0,0207	10,88	21,57	1,98	Up	NM_152647	**C15orf33**	Chromosome 15 open reading frame 33
43	0,000982	0,0207	246,68	370,7	1,50	Up	NM_004783	**TAOK2**	Thousand and one aminoacid kinase 2, transcript variant 1
44	0,001044	0,0207	12,51	3,55	3,52	Down	NR_024560	**LOC100130148**	Hypothetical LOC100130148

45	0,001053	0,0207	563,17	1063,09	1,89	Up	NM_032718	**MFSD9**	Major facilitator superfamily domain containing 9
46	0,001069	0,0207	5,26	11,65	2,21	Up	NM_016944	**TAS2R4**	Taste receptor, type 2, member 4
47	0,001072	0,0207	414	719,2	1,74	Up	NM_002417	**MKI67**	Antigen identified by monoclonal antibody Ki-67, transcript variant 1
48	0,001089	0,0207	56,48	94,75	1,68	Up	NM_014783	**ARHGAP11A**	Rho GTPase activating protein 11A, transcript variant 1
49	0,001141	0,0207	308,41	516,77	1,68	Up	NM_032290	**ANKRD32**	Ankyrin repeat domain 32
50	0,001147	0,0207	5,96	16,16	2,71	Up	NM_005767	**LPAR6**	Lysophosphatidic acid receptor 6, transcript variant 1
51	0,001156	0,0207	5945,84	9051,55	1,52	Up	NM_006696	**BRD8**	Bromodomain containing 8, transcript variant 1
52	0,001190	0,0207	44,83	114,61	2,56	Up	NM_001025195	**CES1**	Carboxylesterase 1 (monocyte/macrophage serine esterase 1), transcript variant 1
53	0,001239	0,0207	33,29	59,6	1,79	Up	NM_138803	**CCDC148**	Coiled-coil domain containing 148, transcript variant 1
54	0,001245	0,0207	7,98	18,8	2,36	Up	NM_001145451	**ARHGEF33**	Rho guanine nucleotide exchange factor (GEF) 33
55	0,001263	0,0207	4,7	29,6	6,30	Up	NM_024727	**LRRC31**	Leucine rich repeat containing 31
56	0,001275	0,0207	4,39	10,98	2,50	Up	NM_015725	**RDH8**	Retinol dehydrogenase 8 (all-trans)
57	0,001290	0,0207	296	489,09	1,65	Up	XR_040889	**LOC729887**	Hypothetical protein LOC729887
58	0,001306	0,0207	8,7	16,33	1,88	Up	NM_152363	**ANKLE1**	Ankyrin repeat and LEM domain containing 1
59	0,001337	0,0207	22,31	5,43	4,11	Down	NM_207416	**FAM75D3**	Family with sequence similarity 75, member D3
60	0,001411	0,0207	929,13	357,09	2,60	Down	NM_002229	**JUNB**	Jun B proto-oncogene
61	0,001440	0,0207	641,11	995,83	1,55	Up	BC022881	**LOC644450**	Hypothetical LOC644450
62	0,001445	0,0207	17,32	4,26	4,06	Down	ENST00000409550	**CTNNA2**	Catenin (cadherin-associated protein), alpha 2, transcript variant 2
63	0,001463	0,0207	63,19	33,52	1,89	Down	XM_001721116	**LOC100131098**	Hypothetical LOC100131098
64	0,001510	0,0207	4,62	9,39	2,03	Up	NM_014410	**CLUL1**	Clusterin-like 1 (retinal), transcript variant 1
65	0,001567	0,0207	5,66	21,44	3,79	Up	NM_007361	**NID2**	Nidogen 2 (osteonidogen)
66	0,001598	0,0207	6,39	4,11	1,55	Down	ENST00000400702	**CCL4L1**	Chemokine (C-C motif) ligand 4-like 1
67	0,001624	0,0207	124,48	80,28	1,55	Down	XR_041976	**LOC727808**	Hypothetical protein LOC727808

68	0,001627	0,0207	7,43	4,01	1,85	Down	XM_001129554	**LOC731414**	Similar to S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))
69	0,001643	0,0207	4,96	21,52	4,34	Up	NM_172387	**NFATC1**	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1, transcript variant 3
70	0,001655	0,0207	150,87	252,24	1,67	Up	NM_024771	**NAA40**	N(alpha)-acetyltransferase 40, NatD catalytic subunit, homolog (*S. cerevisiae*)
71	0,001656	0,0207	5,06	18,25	3,61	Up	NM_014191	**SCN8A**	Sodium channel, voltage gated, type VIII, alpha subunit, transcript variant 1
72	0,001657	0,0207	5,72	3,66	1,56	Down	NM_014619	**GRIK4**	Glutamate receptor, ionotropic, kainate 4
73	0,001663	0,0207	137,23	211,92	1,54	Up	NR_026583	**RACGAP1P**	Rac GTPase activating protein 1 pseudogene
74	0,001696	0,0207	6,4	40,76	6,37	Up	NM_006533	**MIA**	Melanoma inhibitory activity
75	0,001748	0,0207	3241,79	5884,17	1,82	Up	NM_006591	**POLD3**	Polymerase (DNA-directed), delta 3, accessory subunit
76	0,001752	0,0207	585,41	1025,04	1,75	Up	NM_025211	**GKAP1**	G kinase anchoring protein 1, transcript variant 1
77	0,001760	0,0207	21,64	44,81	2,07	Up	NM_032134	**QRICH2**	Glutamine rich 2
78	0,001771	0,0207	9329,11	16472,19	1,77	Up	NM_024786	**ZDHHC11**	Zinc finger, DHHC-type containing 11
79	0,001772	0,0207	4,15	12,45	3,00	Up	NM_030594	**CPEB1**	Cytoplasmic polyadenylation element binding protein 1, transcript variant 1
80	0,001781	0,0207	3185,51	4950,51	1,55	Up	NM_033544	**RCCD1**	RCC1 domain containing 1, transcript variant 1
81	0,001839	0,0207	153,37	338,19	2,21	Up	NM_001039841	**ARHGAP11B**	Rho GTPase activating protein 11B
82	0,001851	0,0207	28553,49	15054,4	1,90	Down	NM_003407	**ZFP36**	Zinc finger protein 36, C3H type, homolog (*M. musculus*)
83	0,001854	0,0207	29,02	44,86	1,55	Up	NM_144709	**PUS10**	Pseudouridylate synthase 10
84	0,001907	0,0208	6,09	13,52	2,22	Up	NM_001007248	**ZNF599**	Zinc finger protein 599
85	0,001933	0,0208	1908,93	1205,73	1,58	Down	NM_004040	**RHOB**	Ras homolog gene family, member B
86	0,001934	0,0208	599,1	966,91	1,61	Up	NM_018369	**DEPDC1B**	DEP domain containing 1B, transcript variant 1
87	0,001944	0,0208	535,71	1043,11	1,95	Up	NM_014959	**CARD8**	Caspase recruitment domain family, member 8, transcript variant 2
88	0,001963	0,0209	6,33	3,93	1,61	Down	NM_005328	**HAS2**	Hyaluronan synthase 2
89	0,001967	0,0209	153,35	232,86	1,52	Up	NM_003470	**USP7**	Ubiquitin specific peptidase 7 (herpes virus-associated)
90	0,001967	0,0209	433,11	655,97	1,51	Up	NM_017941	**C17orf80**	Chromosome 17 open reading frame 80, transcript variant 1

91	0,001979	0,0209	224,6	482,42	2,15	Up	NR_024549	**DMTF1**	Cyclin D binding myb-like transcription factor 1, transcript variant 4
92	0,001999	0,021	5,46	21,32	3,90	Up	NM_022140	**EPB41L4A**	Erythrocyte membrane protein band 4.1 like 4A
93	0,002057	0,0213	42,56	4,1	10,37	Down	NR_027006	**LOC400891**	Chromosome 14 open reading frame 166B pseudogene
94	0,002067	0,0213	80,65	180,83	2,24	Up	NM_152654	**DAND5**	DAN domain family, member 5
95	0,002088	0,0215	11828,33	19351,18	1,64	Up	NM_001042483	**NUPR1**	Nuclear protein, transcriptional regulator, 1, transcript variant 1
96	0,002156	0,0218	795,97	281,27	2,83	Down	NM_001251	**CD68**	CD68 molecule, transcript variant 1
97	0,002158	0,0218	12,31	3,56	3,46	Down	ENST00000375713	**RP11-65D24.2**	HCG2045795 Novel protein
98	0,002183	0,022	2707,91	5648,18	2,09	Up	NM_012291	**ESPL1**	Extra spindle pole bodies homolog 1 (*S. cerevisiae*)
99	0,002192	0,022	265,02	406,7	1,53	Up	NM_058180	**C21orf58**	Chromosome 21 open reading frame 58
100	0,002217	0,0222	219,91	339,94	1,55	Up	NR_021492	**LOC100144603**	Hypothetical transcript
101	0,002248	0,0225	184,95	288,97	1,56	Up	NM_015471	**NSL1**	NSL1, MIND kinetochore complex component, homolog (*S. cerevisiae*), transcript variant 1
102	0,002289	0,0227	922,85	1407,12	1,52	Up	NM_152411	**ZNF786**	Zinc finger protein 786
103	0,002358	0,0233	15162,97	27019,82	1,78	Up	XM_001716434	**ZDHHC11B**	Zinc finger, DHHC-type containing 11B
104	0,002366	0,0233	28	576,68	20,60	Up	NM_000721	**CACNA1E**	Calcium channel, voltage-dependent, R type, alpha 1E subunit
105	0,002417	0,0236	150,91	259,22	1,72	Up	NM_018248	**NEIL3**	Nei endonuclease VIII-like 3 (*E. coli*)
106	0,002525	0,0244	1402,7	584,74	2,40	Down	NM_001276	**CHI3L1**	Chitinase 3-like 1 (cartilage glycoprotein-39)
107	0,002644	0,0253	148,81	98,41	1,51	Down	NM_001145724	**HOMER3**	Homer homolog 3 (*Drosophila*), transcript variant 4
108	0,002668	0,0255	5,23	3,35	1,56	Down	NM_005408	**CCL13**	Chemokine (C-C motif) ligand 13
109	0,002707	0,0258	115,09	233,1	2,03	Up	NM_022346	**NCAPG**	Non-SMC condensin I complex, subunit G
110	0,002751	0,0262	353,44	534,17	1,51	Up	NM_017952	**PTCD3**	Pentatricopeptide repeat domain 3, nuclear gene encoding mitochondrial protein
111	0,002753	0,0262	9,37	20,4	2,18	Up	ENST00000414093	**AC120053.1**	Putative uncharacterized protein FP6628
112	0,002758	0,0262	428,37	736,43	1,72	Up	NR_029410	**LOC389765**	Kinesin family member 27 pseudogene
113	0,002814	0,0266	242,48	379,15	1,56	Up	NM_032329	**ING5**	Inhibitor of growth family, member 5

114	0,002817	0,0266	371,27	620,21	1,67	Up	NR_027329	**KIAA1908**	Hypothetical LOC114796, transcript variant 1
115	0,002826	0,0267	956,65	549,23	1,74	Down	NM_178865	**SERINC2**	Serine incorporator 2
116	0,002834	0,0268	16,73	26,52	1,59	Up	NM_032918	**RERG**	RAS-like, estrogen-regulated, growth inhibitor, transcript variant 1
117	0,002913	0,0274	16763,15	7767,94	2,16	Down	NM_002228	**JUN**	Jun proto-oncogene
118	0,002930	0,0275	2733,65	4820,04	1,76	Up	NM_001067	**TOP2A**	Topoisomerase (DNA) II alpha 170kDa
119	0,002956	0,0278	27,23	43,46	1,60	Up	NM_015562	**UBXN7**	UBX domain protein 7
120	0,002960	0,0278	212,9	381,17	1,79	Up	NM_001009812	**LBX2**	Ladybird homeobox 2
121	0,002984	0,028	65,75	189,95	2,89	Up	ENST00000370964	**DEPDC1**	DEP domain-containing 1 protein 1A
122	0,003025	0,0283	382,8	652,34	1,70	Up	NM_003483	**HMGA2**	High mobility group AT-hook 2, transcript variant 1
123	0,003033	0,0283	5,49	11,62	2,12	Up	NM_153608	**ZNF114**	Zinc finger protein 114
124	0,003058	0,0286	390,04	604,64	1,55	Up	NM_016195	**KIF20B**	Kinesin family member 20B
125	0,003065	0,0286	139,49	220,03	1,58	Up	NM_014810	**CEP350**	Centrosomal protein 350kDa
126	0,003071	0,0286	446,61	687,95	1,54	Up	NM_019607	**C8orf44**	Chromosome 8 open reading frame 44
127	0,003100	0,0289	247,61	133,3	1,86	Down	NM_016518	**PIPOX**	Pipecolic acid oxidase
128	0,003187	0,0296	146,56	89,64	1,64	Down	NM_021995	**UTS2**	Urotensin 2, transcript variant 1
129	0,003244	0,0301	48,05	117,01	2,44	Up	XM_001723077	**LOC100132934 (TDGF2)**	Similar to hCG1644233 (teratocarcinoma-derived growth factor 2, pseudogene)
130	0,003276	0,0303	18,86	43,05	2,28	Up	NM_001822	**CHN1**	Chimerin (chimaerin) 1, transcript variant 1
131	0,003401	0,0314	39,16	19,78	1,98	Down	NM_001012288	**CRLF2**	Cytokine receptor-like factor 2, transcript variant 2
132	0,003520	0,0323	25,35	108,36	4,27	Up	NM_052813	**CARD9**	Caspase recruitment domain family, member 9, transcript variant 1
133	0,003528	0,0324	535,87	281,82	1,90	Down	NM_199460	**CACNA1C**	Calcium channel, voltage-dependent, L type, alpha 1C subunit, transcript variant 1
134	0,003533	0,0324	17,11	32,5	1,90	Up	NM_015272	**RPGRIP1L**	RPGRIP1-like, transcript variant 1
135	0,003546	0,0325	4,46	12,14	2,72	Up	ENST00000399442	**CRYZL1**	Crystallin, zeta (quinone reductase)-like 1
136	0,003585	0,0328	225,85	368,5	1,63	Up	NM_020897	**HCN3**	Hyperpolarization activated cyclic nucleotide-gated potassium channel 3

137	0,003615	0,0331	10,91	3,54	3,08	Down	NM_001005189	**OR6Y1**	Olfactory receptor, family 6, subfamily Y, member 1
138	0,003635	0,0333	392,42	152,22	2,58	Down	NM_006169	**NNMT**	Nnicotinamide N-methyltransferase
139	0,003664	0,0335	1043,98	293,4	3,56	Down	NM_016084	**RASD1**	RAS, dexamethasone-induced 1
140	0,003784	0,0345	505,68	772,18	1,53	Up	NM_001039618	**CREBZF**	CREB/ATF bZIP transcription factor, transcript variant 1
141	0,003876	0,0352	21,26	35,8	1,68	Up	NM_001024596	**ZNF772**	Zinc finger protein 772, transcript variant 1
142	0,003880	0,0352	24,04	77,21	3,21	Up	NM_000064	**C3**	Complement component 3
143	0,003884	0,0352	28,64	74,79	2,61	Up	NM_001135187	**AGFG1**	ArfGAP with FG repeats 1, transcript variant 1
144	0,003898	0,0354	296	497,1	1,68	Up	NM_001031801	**LIMK2**	LIM domain kinase 2, transcript variant 1
145	0,003902	0,0354	4,41	7,51	1,70	Up	NM_001008226	**FAM154B**	Family with sequence similarity 154, member B
146	0,003973	0,036	728,93	1106,6	1,52	Up	NM_012249	**RHOQ**	Ras homolog gene family, member Q
147	0,003979	0,036	20342,9	10372,63	1,96	Down	NM_003254	**TIMP1**	TIMP metallopeptidase inhibitor 1
148	0,003996	0,0361	614,69	995,63	1,62	Up	NM_001048166	**STIL**	SCL/TAL1 interrupting locus, transcript variant 1
149	0,004005	0,0362	5,42	11,79	2,18	Up	NM_144648	**LRGUK**	Leucine-rich repeats and guanylate kinase domain containing
150	0,004009	0,0362	39,63	62,33	1,57	Up	NM_006733	**CENPI**	Centromere protein I
151	0,004020	0,0363	1011,36	1847,69	1,83	Up	NM_207418	**FAM72D**	Family with sequence similarity 72, member D
152	0,004021	0,0363	4,79	7,79	1,63	Up	NM_000906	**NPR1**	Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)
153	0,004043	0,0365	3231,64	1732,04	1,87	Down	NM_004751	**GCNT3**	Glucosaminyl (N-acetyl) transferase 3, mucin type
154	0,004056	0,0366	31,53	59,21	1,88	Up	NR_024420	**LOC389634**	Hypothetical LOC389634
155	0,004087	0,0368	4,73	8,07	1,71	Up	XM_001726774	**LOC100133405**	Hypothetical LOC100133405
156	0,004109	0,037	26,39	76,24	2,89	Up	NM_203307	**ZNF321**	Zinc finger protein 321
157	0,004128	0,0372	13,33	29,95	2,25	Up	NM_001134233	**C1orf204**	Chromosome 1 open reading frame 204
158	0,004161	0,0375	4,9	8,85	1,81	Up	ENST00000399191	**CBR1**	Carbonyl reductase 1
159	0,004167	0,0375	834,89	1571,99	1,88	Up	NM_001123168	**FAM72A**	Family with sequence similarity 72, member A

160	0,004233	0,038	63,74	119,82	1,88	Up	XR_041071	**LOC646719**	Hypothetical LOC646719
161	0,004253	0,0382	5,19	14,53	2,80	Up	NM_005491	**MAMLD1**	Mastermind-like domain containing 1, transcript variant 2
162	0,004300	0,0386	44,05	92,88	2,11	Up	NM_001077238	**SPPL2B**	Signal peptide peptidase-like 2B, transcript variant 3
163	0,004319	0,0387	253,25	648,23	2,56	Up	NM_017563	**IL17RD**	Interleukin 17 receptor D
164	0,004332	0,0388	20,28	33,72	1,66	Up	NM_020322	**ACCN3**	Amiloride-sensitive cation channel 3, transcript variant 3
165	0,004388	0,0393	692,1	1266,19	1,83	Up	ENST00000440429	**AC006427.1**	–
166	0,004420	0,0395	179,68	720,47	4,01	Up	NM_003212	**TDGF1**	Teratocarcinoma-derived growth factor 1, transcript variant 1
167	0,004490	0,0401	2489,15	4280,1	1,72	Up	NM_001025202	**STAG3L2**	Stromal antigen 3-like 2
168	0,004518	0,0403	41,72	75,03	1,80	Up	NM_002202	**ISL1**	ISL LIM homeobox 1
169	0,004572	0,0408	129,06	240,04	1,86	Up	ENST00000333129	**FIGN**	Fidgetin
170	0,004620	0,0411	421,02	664,26	1,58	Up	NM_134269	**SMTN**	Smoothelin, transcript variant 2
171	0,004668	0,0416	19,82	41,09	2,07	Up	NM_134425	**SLC26A1**	Solute carrier family 26 (sulfate transporter), member 1, transcript variant 2
172	0,004724	0,042	5,78	17,19	2,97	Up	NM_153819	**RASGRP2**	RAS guanyl releasing protein 2 (calcium and DAG-regulated), transcript variant 2
173	0,004731	0,042	4,31	22,38	5,19	Up	NM_003505	**FZD1**	Frizzled homolog 1 (*Drosophila*)
174	0,004749	0,0422	5,88	10,61	1,80	Up	ENST00000366845	**AC092811.1**	Putative uncharacterized protein C1orf197
175	0,004820	0,0428	5,97	10,36	1,74	Up	NR_024385	**LOC151300**	Hypothetical LOC151300, transcript variant 1
176	0,004834	0,0428	4445,73	7058,18	1,59	Up	NM_007174	**CIT**	Citron (rho-interacting, serine/threonine kinase 21)
177	0,004854	0,043	303,64	501,46	1,65	Up	NM_005069	**SIM2**	Single-minded homolog 2 (*Drosophila*), transcript variant SIM2
178	0,004903	0,0434	152,16	284,99	1,87	Up	ENST00000317419	**RABGAP1**	RAB GTPase activating protein 1
179	0,004936	0,0436	40,22	82,29	2,05	Up	NM_018338	**WDR52**	WD repeat domain 52, transcript variant 2
180	0,004991	0,044	40,2	61,64	1,53	Up	NM_001080209	**TMEM200C**	Transmembrane protein 200C
181	0,005040	0,0444	289,63	182,84	1,58	Down	NM_016275	**SELT**	Selenoprotein T
182	0,005092	0,0448	4816,18	2371,21	2,03	Down	NM_001425	**EMP3**	Epithelial membrane protein 3

183	0,005098	0,0449	35,89	62,04	1,73	Up	NM_052843	**OBSCN**	Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF, transcript variant 1
184	0,005237	0,046	6,43	13,16	2,05	Up	NR_001281	**PCDHB18**	Protocadherin beta 18 pseudogene
185	0,005240	0,046	106,82	209,94	1,97	Up	NM_174942	**GAS2L3**	Growth arrest-specific 2 like 3
186	0,005260	0,0461	16809,28	3613,96	4,65	Down	NM_005252	**FOS**	FBJ murine osteosarcoma viral oncogene homolog
187	0,005268	0,0462	73,24	137,99	1,88	Up	NM_182566	**VMO1**	Vitelline membrane outer layer 1 homolog (*G. galus*), transcript variant 1
188	0,005269	0,0462	65,26	128,75	1,97	Up	NM_001014975	**CFH**	Complement factor H, transcript variant 2
189	0,005275	0,0462	3672,54	11018,29	3,00	Up	NM_031942	**CDCA7**	Cell division cycle associated 7, transcript variant 1
190	0,005325	0,0466	5,26	328,55	62,46	Up	NM_006418	**OLFM4**	Olfactomedin 4
191	0,005330	0,0466	1463,74	2490,93	1,70	Up	NM_020937	**FANCM**	Fanconi anemia, complementation group M
192	0,005330	0,0466	1021,99	1593,65	1,56	Up	NM_005664	**MKRN3**	Makorin ring finger protein 3
193	0,005363	0,0469	2739,14	4156,73	1,52	Up	NM_012279	**ZNF346**	Zinc finger protein 346
194	0,005363	0,0469	11,08	26,23	2,37	Up	NM_001130404	**PRR20B**	Proline rich 20B
195	0,005368	0,0469	5334,37	8220,38	1,54	Up	NM_013355	**PKN3**	Protein kinase N3
196	0,005391	0,0471	4,25	10,81	2,54	Up	NM_138732	**NRXN2**	Neurexin 2, transcript variant alpha-2
197	0,005424	0,0473	10,64	38,16	3,59	Up	NM_018430	**TSNAXIP1**	Translin-associated factor X interacting protein 1
198	0,005450	0,0476	1533,15	2372,14	1,55	Up	NM_020242	**KIF15**	Kinesin family member 15
199	0,005496	0,0479	44,9	74,81	1,67	Up	NM_178504	**DNAH12**	Dynein, axonemal, heavy chain 12, transcript variant 1
200	0,005497	0,0479	211,54	358,48	1,69	Up	NR_015363	**MGC21881**	Hypothetical locus MGC21881
201	0,005561	0,0485	15,92	8,59	1,85	Down	NM_024590	**ARSJ**	Arylsulfatase family, member J
202	0,005643	0,0491	6,88	19,57	2,84	Up	NM_005840	**SPRY3**	Sprouty homolog 3 (*Drosophila*)
203	0,005660	0,0493	37,15	63,89	1,72	Up	XM_001718573	**LOC100133176**	Similar to zinc finger protein 195
204	0,005721	0,0497	12,28	22,38	1,82	Up	NM_000453	**SLC5A5**	Solute carrier family 5 (sodium iodide symporter), member 5
205	0,005803	0,0504	4168,79	6337,51	1,52	Up	NM_138555	**KIF23**	Kinesin family member 23, transcript variant 1

206	0,005834	0,0506	11,44	5,59	2,05	Down	ENST00000375389	**FAM120A**	Family with sequence similarity 120A
207	0,005840	0,0507	8,17	14,92	1,83	Up	NM_001030005	**CPLX3**	Complexin 3
208	0,005850	0,0507	5,64	17,53	3,11	Up	NM_014817	**TRIL**	TLR4 interactor with leucine-rich repeats
209	0,005905	0,0512	17204,13	10191,62	1,69	Down	NM_003945	**ATP6V0E1**	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e1
210	0,005927	0,0513	195,39	343,53	1,76	Up	NM_000933	**PLCB4**	Phospholipase C, beta 4, transcript variant 1
211	0,005935	0,0513	4,39	10,68	2,43	Up	BC045815	**LOC541467**	Hypothetical LOC541467, (cDNA clone IMAGE:4830703), partial cds (testis)
212	0,005956	0,0515	924,26	1477,5	1,60	Up	NM_032193	**RNASEH2C**	Ribonuclease H2, subunit C
213	0,005972	0,0516	160,19	299,38	1,87	Up	ENST00000398305	**AC091132.4**	LOC652203 protein
214	0,005981	0,0517	491,44	2602,08	5,29	Up	NM_004822	**NTN1**	Netrin 1
215	0,006021	0,052	46,8	102,81	2,20	Up	NM_012253	**TKTL1**	Transketolase-like 1, transcript variant 1
216	0,006045	0,0522	16,95	26,85	1,58	Up	NM_006044	**HDAC6**	Histone deacetylase 6
217	0,006054	0,0522	804,77	1309,32	1,63	Up	BC017694	**FAM128B**	Family with sequence similarity 128, member B (cDNA clone IMAGE:4415420), with apparent retained intron (Kidney, hypernephroma)
218	0,006083	0,0524	9241,76	14123,7	1,53	Up	XR_078993	**LOC100293090**	Similar to DC24, pseudogene
219	0,006100	0,0526	397,29	607,63	1,53	Up	NM_153358	**ZNF791**	Zinc finger protein 791
220	0,006223	0,0536	13,07	52,02	3,98	Up	ENST00000372583	**HIVEP3**	Human immunodeficiency virus type I enhancer binding protein 3
221	0,006255	0,0538	37,82	22,98	1,65	Down	NM_002962	**S100A5**	S100 calcium binding protein A5
222	0,006292	0,0541	38120,18	24575,73	1,55	Down	NM_004261	**15-Set**	15 kDa selenoprotein, transcript variant 1
223	0,006322	0,0543	103,91	219,15	2,11	Up	NM_000641	**IL11**	Interleukin 11
224	0,006324	0,0543	21,29	50,55	2,37	Up	NM_001670	**ARVCF**	Armadillo repeat gene deleted in velocardiofacial syndrome
225	0,006393	0,0548	5,44	14,72	2,71	Up	NM_004321	**KIF1A**	Kinesin family member 1A
226	0,006421	0,055	5,83	15,01	2,57	Up	NM_003469	**SCG2**	Secretogranin II
227	0,006445	0,0551	179,98	290,33	1,61	Up	NM_002604	**PDE7A**	Phosphodiesterase 7A, transcript variant 2
228	0,006484	0,0555	141,93	256,71	1,81	Up	NM_001039083	**ARL17B**	ADP-ribosylation factor-like 17B, transcript variant 1

229	0,006528	0,0558	7,38	17,4	2,36	Up	NM_015077	**SARM1**	Sterile alpha and TIR motif containing 1
230	0,006579	0,0561	24,64	9,86	2,50	Down	NM_144598	**LRRC28**	Leucine rich repeat containing 28
231	0,006616	0,0564	64,11	103,48	1,61	Up	NM_017915	**C12orf48**	Chromosome 12 open reading frame 48
232	0,006629	0,0565	432,81	700,71	1,62	Up	NM_014844	**TECPR2**	Tectonin beta-propeller repeat containing 2, transcript variant 1
233	0,006644	0,0566	24,72	49,38	2,00	Up	NM_017790	**RGS3**	Regulator of G-protein signaling 3, transcript variant 3
234	0,006657	0,0567	200,93	319,12	1,59	Up	NM_022768	**RBM15**	RNA binding motif protein 15
235	0,006661	0,0567	1199,09	1875,49	1,56	Up	NM_194249	**DND1**	Dead end homolog 1 (*D. rerio*)
236	0,006667	0,0567	478,2	720,63	1,51	Up	NM_014827	**ZC3H11A**	Zinc finger CCCH-type containing 11A
237	0,006686	0,0569	48451,05	27025,98	1,79	Down	NM_182744	**NBL1**	Neuroblastoma, suppression of tumorigenicity 1, transcript variant 1
238	0,006709	0,057	5,49	14,9	2,71	Up	ENST00000373815	**ANK3**	Ankyrin 3, node of Ranvier (ankyrin G)
239	0,006729	0,0572	313,67	551,1	1,76	Up	NR_024270	**LOC400752**	Hypothetical LOC400752
240	0,006806	0,0577	404,05	742,6	1,84	Up	ENST00000409310	**PLGLB1**	Plasminogen-like B1 (subjected to nonsense mediated decay)
241	0,006842	0,058	7,53	13,7	1,82	Up	ENST00000298858	**C14orf159**	UPF0317 protein C14orf159, mitochondrial
242	0,006887	0,0583	7,16	16,97	2,37	Up	ENST00000371799	**OLFM1**	Olfactomedin 1
243	0,006922	0,0586	2277,06	798,28	2,85	Down	NM_004417	**DUSP1**	Dual specificity phosphatase 1
244	0,006931	0,0586	7,6	30,02	3,95	Up	NM_021023	**CFHR3**	Complement factor H-related 3, transcript variant 1
245	0,006939	0,0586	1251,13	1906,15	1,52	Up	NM_133330	**WHSC1**	Wolf-Hirschhorn syndrome candidate 1, transcript variant 1
246	0,006942	0,0586	56,8	100,78	1,77	Up	XM_001126849	**LOC727900**	Hypothetical LOC727900
247	0,006946	0,0587	112,31	185,92	1,66	Up	NM_015185	**ARHGEF9**	Cdc42 guanine nucleotide exchange factor (GEF) 9, transcript variant 1
248	0,006989	0,0589	4,74	8,96	1,89	Up	NM_001040462	**BTNL8**	Butyrophilin-like 8, transcript variant 2
249	0,007091	0,0597	108,56	181,78	1,67	Up	NR_026778	**NCRNA00201**	Non-protein coding RNA 201
250	0,007097	0,0598	622,98	1085,72	1,74	Up	NM_004536	**NAIP**	NLR family, apoptosis inhibitory protein, transcript variant 1
251	0,007163	0,0603	102,18	165,94	1,62	Up	NM_004279	**PMPCB**	Peptidase (mitochondrial processing) beta, nuclear gene encoding mitochondrial protein

252	0,007186	0,0604	157,98	284	1,80	Up	NM_007018	**CEP110**	Centrosomal protein 110kDa
253	0,007199	0,0605	230,04	463,89	2,02	Up	NM_014226	**RAGE**	Renal tumor antigen
254	0,007245	0,0608	24	80,7	3,36	Up	ENST00000397236	**RNF144B**	Ring finger protein 144B
255	0,007257	0,0609	49,44	85,29	1,73	Up	NM_032221	**CHD6**	Chromodomain helicase DNA binding protein 6
256	0,007284	0,0611	102,17	197,04	1,93	Up	NM_000108	**DLD**	Dihydrolipoamide dehydrogenase
257	0,007301	0,0613	4,45	8,1	1,82	Up	NM_021046	**KRTAP5-8**	Keratin associated protein 5-8
258	0,007313	0,0613	242,27	369,38	1,52	Up	NM_013320	**HCFC2**	Host cell factor C2
259	0,007351	0,0616	124,34	227,28	1,83	Up	NM_001012969	**ADAL**	Adenosine deaminase-like, transcript variant 2
260	0,007355	0,0616	7,06	14,02	1,99	Up	ENST00000369086	**TIAL1**	TIA1 cytotoxic granule-associated RNA binding protein-like 1
261	0,007356	0,0616	112,13	418,4	3,73	Up	NM_152359	**CPT1C**	Carnitine palmitoyltransferase 1C, transcript variant 2
262	0,007392	0,0619	173,58	341,88	1,97	Up	NM_052971	**LEAP2**	Liver expressed antimicrobial peptide 2
263	0,007403	0,062	41,12	85,88	2,09	Up	NM_013267	**GLS2**	Glutaminase 2 (liver, mitochondrial), nuclear gene encoding mitochondrial protein
264	0,007414	0,062	5,07	8,34	1,64	Up	XM_001720272	**LOC100131436**	Hypothetical LOC100131436
265	0,007417	0,062	749,8	1216,79	1,62	Up	NM_003503	**CDC7**	Cell division cycle 7 homolog (*S. cerevisiae*), transcript variant 1
266	0,007485	0,0626	6228,86	2829,01	2,20	Down	NM_002178	**IGFBP6**	Insulin-like growth factor binding protein 6
267	0,007561	0,0632	5,46	10,16	1,86	Up	NM_144727	**CRYGN**	Crystallin, gamma N
268	0,007572	0,0632	79,65	149,35	1,88	Up	NM_001080519	**BAHCC1**	BAH domain and coiled-coil containing 1
269	0,007577	0,0633	7,68	15,16	1,97	Up	NM_002471	**MYH6**	Myosin, heavy chain 6, cardiac muscle, alpha
270	0,007608	0,0635	27,64	85,68	3,10	Up	NM_152709	**STOX1**	Storkhead box 1, transcript variant 1
271	0,007628	0,0636	17,98	28,8	1,60	Up	NM_001003699	**RREB1**	Ras responsive element binding protein 1, transcript variant 1
272	0,007639	0,0637	11,05	25,5	2,31	Up	NR_026991	**C3orf47**	Chromosome 3 open reading frame 47
273	0,007671	0,0639	497,58	807,87	1,62	Up	NM_173690	**SCAI**	Suppressor of cancer cell invasion, transcript variant 1
274	0,007738	0,0644	15,46	50,19	3,25	Up	NM_005618	**DLL1**	Delta-like 1 (*Drosophila*)

275	0,007775	0,0647	80,38	152,9	1,90	Up	NR_027302	**XPA**	Xeroderma pigmentosum, complementation group A, transcript variant 2
276	0,007872	0,0654	187,17	302,01	1,61	Up	NM_014915	**ANKRD26**	Ankyrin repeat domain 26
277	0,007913	0,0657	72,79	131,62	1,81	Up	ENST00000400890	**AC011043.1**	Putative uncharacterized protein DKFZp667B1610
278	0,007926	0,0658	664,9	1021,98	1,54	Up	NM_207333	**ZNF320**	Zinc finger protein 320
279	0,007937	0,0658	61,18	147,34	2,41	Up	NR_026960	**C21orf125**	Chromosome 21 open reading frame 125
280	0,007967	0,066	3890,31	2013,48	1,93	Down	NM_000716	**C4BPB**	Complement component 4 binding protein, beta, transcript variant 1
281	0,007983	0,0661	10,66	32,01	3,00	Up	ENST00000380735	**SHROOM3**	Shroom family member 3
282	0,007999	0,0662	5,62	3,41	1,65	Down	NM_198956	**SP8**	Sp8 transcription factor, transcript variant 2
283	0,008040	0,0665	4,17	6,69	1,60	Up	XR_038842	**LOC642943**	Hypothetical LOC642943 (family with sequence similarity 75, member A1 pseudogene)
284	0,008046	0,0666	20302,64	31351,6	1,54	Up	NM_182914	**SYNE2**	Spectrin repeat containing, nuclear envelope 2, transcript variant 5
285	0,008059	0,0666	53,67	95,97	1,79	Up	NM_001142579	**ZNF780A**	Zinc finger protein 780A, transcript variant 4
286	0,008081	0,0668	6,45	4,04	1,60	Down	NM_152680	**TMEM154**	Transmembrane protein 154
287	0,008092	0,0669	1121,58	2127,72	1,90	Up	NM_014875	**KIF14**	Kinesin family member 14
288	0,008094	0,0669	39,02	191,7	4,91	Up	NM_139022	**TSPAN32**	Tetraspanin 32
289	0,008134	0,0672	8,93	24,96	2,80	Up	ENST00000400340	**AL139349.1**	Putative uncharacterized protein ENSP00000383194
290	0,008159	0,0673	204,84	448,15	2,19	Up	NM_031945	**TSPAN10**	Tetraspanin 10
291	0,008222	0,0677	3436,39	8403,23	2,45	Up	NR_002569	**SCARNA9**	Small Cajal body-specific RNA 9, guide RNA
292	0,008240	0,0679	62,91	105,66	1,68	Up	NR_029401	**LOC731275**	Hypothetical LOC731275
293	0,008245	0,0679	92,2	153,95	1,67	Up	NM_145023	**CCDC7**	Coiled-coil domain containing 7, transcript variant 1
294	0,008258	0,0679	5,7	12,3	2,16	Up	ENST00000440451	**CCDC162**	Coiled-coil domain containing 162 pseudogene
295	0,008259	0,0679	5,84	15,21	2,60	Up	ENST00000394565	**AKAP9**	A kinase (PRKA) anchor protein (yotiao) 9
296	0,008305	0,0683	177,2	277,8	1,57	Up	ENST00000405673	**FAM118A**	Family with sequence similarity 118, member A
297	0,008306	0,0683	45,98	71,03	1,54	Up	NM_025081	**NYNRIN**	NYN domain and retroviral integrase containing

298	0,008324	0,0684	579,61	2318	4,00	Up	NM_005012	**ROR1**	Receptor tyrosine kinase-like orphan receptor 1, transcript variant 1
299	0,008387	0,0688	1897,08	2937,82	1,55	Up	NR_029407	**LOC642361**	Hypothetical LOC642361
300	0,008397	0,0689	2379,2	4880,21	2,05	Up	NM_001813	**CENPE**	Centromere protein E, 312kDa
301	0,008417	0,069	5,23	11,66	2,23	Up	XM_001713897	**LOC100131594 (GUSBP1)**	Hypothetical protein LOC100131594 (glucuronidase, beta pseudogene 1)
302	0,008479	0,0695	12,93	32,24	2,49	Up	ENST00000453599	**RP11-528L24.3**	Putative uncharacterized protein ENSP00000395818
303	0,008540	0,07	428,2	755,3	1,76	Up	NM_000387	**SLC25A20**	Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20, nuclear gene encoding mitochondrial protein
304	0,008561	0,0701	71,9	169,98	2,36	Up	NM_023921	**TAS2R10**	Taste receptor, type 2, member 10
305	0,008586	0,0703	3214,77	662,03	4,86	Down	NM_005554	**KRT6A**	Keratin 6A
306	0,008602	0,0704	4278,55	6630,99	1,55	Up	NM_145697	**NUF2**	NUF2, NDC80 kinetochore complex component, homolog (*S. cerevisiae*), transcript variant 1
307	0,008616	0,0705	3525,77	5819,24	1,65	Up	NM_203488	**ACYP1**	Acylphosphatase 1, erythrocyte (common) type, transcript variant 2
308	0,008666	0,0708	184,7	352,13	1,91	Up	NM_130773	**CNTNAP5**	Contactin associated protein-like 5
309	0,008683	0,0709	9,13	16	1,75	Up	ENST00000399256	**APOB**	Apolipoprotein B (including Ag(x) antigen)
310	0,008701	0,0711	7,56	24,95	3,30	Up	NM_020945	**WDFY4**	WDFY family member 4
311	0,008710	0,0711	80,36	135,13	1,68	Up	NM_020347	**LZTFL1**	Leucine zipper transcription factor-like 1
312	0,008738	0,0713	5,59	10,96	1,96	Up	NM_175619	**ZAR1**	Zygote arrest 1
313	0,008787	0,0717	584,45	1059,22	1,81	Up	NM_016343	**CENPF**	Centromere protein F, 350/400kDa (mitosin)
314	0,008814	0,0719	47,5	76,96	1,62	Up	NM_032814	**RNFT2**	Ring finger protein, transmembrane 2, transcript variant 2
315	0,008828	0,072	1821,11	4038,96	2,22	Up	NM_018136	**ASPM**	Asp (abnormal spindle) homolog, microcephaly associated (*Drosophila*)
316	0,008839	0,072	62,36	10,39	6,00	Down	NM_025048	**GPR110**	G protein-coupled receptor 110, transcript variant 2
317	0,008875	0,0723	13,41	21,88	1,63	Up	NM_182589	**HTR3E**	5-hydroxytryptamine (serotonin) receptor 3, family member E
318	0,008897	0,0724	5,29	3,24	1,63	Down	NM_000236	**LIPC**	Lipase, hepatic
319	0,008911	0,0725	87,95	29,84	2,95	Down	NM_005110	**GFPT2**	Glutamine-fructose-6-phosphate transaminase 2
320	0,009018	0,0732	787,28	1224,48	1,56	Up	NM_001129899	**ZNF673**	Zinc finger family member 673, transcript variant 3

321	0,009128	0,074	329,6	539,58	1,64	Up	ENST00000372560	**PPCS**	Phosphopantothenoylcysteine synthetase
322	0,009146	0,0741	132,64	80,32	1,65	Down	NM_012337	**CCDC19**	Coiled-coil domain containing 19
323	0,009163	0,0742	1370,51	2580,97	1,88	Up	NM_001023587	**ABCC5**	ATP-binding cassette, sub-family C (CFTR/MRP), member 5, transcript variant 2
324	0,009282	0,075	33,87	57,19	1,69	Up	ENST00000332454	**AC020928.3**	Putative zinc finger protein ENSP00000330994
325	0,009337	0,0754	556,36	887,16	1,59	Up	NM_198458	**ZNF497**	Zinc finger protein 497
326	0,009372	0,0756	383,24	807,5	2,11	Up	ENST00000340621	**CCDC144A**	Coiled-coil domain containing 144A
327	0,009417	0,0759	95,23	41,41	2,30	Down	XM_001132797	**LOC388630**	UPF0632 protein A
328	0,009459	0,0762	94,2	283,67	3,01	Up	NM_003048	**SLC9A2**	Solute carrier family 9 (sodium/hydrogen exchanger), member 2
329	0,009512	0,0765	56,07	85,62	1,53	Up	NR_029192	**LOC100133315**	Transient receptor potential cation channel, subfamily C, member 2-like
330	0,009564	0,0769	4,19	14,72	3,51	Up	NM_017826	**SOHLH2**	Spermatogenesis and oogenesis specific basic helix-loop-helix 2
331	0,009589	0,0771	4,91	15,78	3,21	Up	NM_005608	**PTPRCAP**	Protein tyrosine phosphatase, receptor type, C-associated protein
332	0,009620	0,0773	16,56	26,55	1,60	Up	NM_002739	**PRKCG**	Protein kinase C, gamma
333	0,009630	0,0774	14,4	34,63	2,40	Up	NM_021151	**CROT**	Carnitine O-octanoyltransferase, transcript variant 2
334	0,009634	0,0774	108,88	185,45	1,70	Up	NM_182498	**ZNF428**	Zinc finger protein 428
335	0,009725	0,078	32608,24	18768,07	1,74	Down	NM_001039492	**FHL2**	Four and a half LIM domains 2, transcript variant 5
336	0,009816	0,0787	5,91	11,21	1,90	Up	ENST00000301679	**ITFG3**	Integrin alpha FG-GAP repeat containing 3
337	0,009829	0,0788	5,47	9,99	1,83	Up	NM_139021	**MAPK15**	Mitogen-activated protein kinase 15
338	0,009901	0,0793	6,99	4,44	1,57	Down	NM_032523	**OSBPL6**	Oxysterol binding protein-like 6, transcript variant 1
339	0,009944	0,0796	7,03	14,83	2,11	Up	NR_028389	**LOC100128640**	Hypothetical LOC100128640
340	0,009964	0,0796	56,08	115,72	2,06	Up	NM_144641	**PPM1M**	Protein phosphatase, Mg2+/Mn2+ dependent, 1M, transcript variant 1