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MINIREVIEWS

# Extending the functions of the homeotic transcription factor Cdx2 in the digestive system through nontranscriptional activities

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## Abstract

The homeoprotein encoded by the intestinal-specific *Cdx2* gene is a major regulator of gut development and homeostasis, also involved in colon cancer as well as in intestinal-type metaplasias when it is abnormally expressed outside the gut. At the molecular level, structure/function studies have demonstrated that the Cdx2 protein is a transcription factor containing a conserved homeotic DNA-binding domain made of three alpha helixes arranged in a helix-turn-helix motif, preceded by a transcriptional domain and followed by a regulatory domain. The protein interacts with several thousand sites on the chromatin and widely regulates intestinal functions in stem/progenitor cells as well as in mature differentiated cells. Yet, this transcription factor also acts trough original nontranscriptional mechanisms. Indeed, the identification of novel protein partners of Cdx2 and also of a splicing variant revealed unexpected functions in the control of signaling pathways like the Wnt and NF-κB pathways, in double-strand break DNA repair and in premessenger RNA splicing. These novel functions of Cdx2 must be considered to fully understand the complexity of the role of Cdx2 in the healthy intestine and in diseases.

**Key words:** Homeobox; Transcription; Signaling; DNA repair; RNA splicing; Intestine; Development; Cancer; Homeostasis



**Core tip:** The homeobox gene *Cdx2* plays crucial functions in the gut, being the one determinant of intestinal identity during embryonic development, and then a key regulator of homeostasis of the gut epithelium throughout adulthood. It acts at the level of the stem/progenitor cells as well as in mature epithelial cells. *Cdx2* is also important in digestive diseases, especially in colorectal cancer where it is thought to have a tumor suppressor role. In addition, it becomes abnormally expressed ectopically in non-intestinal organs, including gastric intestinal-type metaplasia and Barrett's esophagus and their related adenocarcinomas, and even in leukemia. The homeoprotein Cdx2 primarily acts as a DNA-binding transcription factor. However, recent reports provide evidence for novel mechanisms of action that are transcription-independent. In this

review, we summarize these new data that considerably extend the molecular potential of Cdx2 and open new questions and research area regarding the role of this homeoprotein in physiology and pathology.

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## *CDX2*: A MAJOR DETERMINANT OF INTESTINAL DEVELOPMENT AND HOMEOSTASIS, ALSO CONTRIBUTING TO DIGESTIVE DISEASES

Homeobox genes, first identified in pioneer studies conducted in *Drosophila melanogaster*, encode evolutionary-conserved transcription factors that are key players of the embryonic development, especially for axis formation and patterning. Some of them remain expressed throughout adulthood to control tissue homeostasis, and therefore they might be involved in diseases. Although many homeobox genes are dispersed in the genome, others are clustered. Mammals have four *Hox* gene clusters, the clusters *HoxA* to *HoxD* gathering 9 to 11 genes, as well as one *paraHox* cluster made of 3 homeobox genes: *Gsx1*, *Pdx1* and *Cdx2* (*Caudal*-related homeobox); two additional *Cdx* genes are dispersed, *Cdx1* and *Cdx4* (see Ref 1 for a review of the organization and expression of homeobox genes). Here, we will focus on the *paraHox* homeobox gene *Cdx2*[1].

The homeobox gene *Cdx2* exhibits a complex expression pattern described in detail in mice. It turns on in 8- to 32-cells zygotes to progressively concentrate in the trophectoderm[2]. Embryonic tissues first express *Cdx2* after gastrulation around E8.5 at the level of the tail bud in the three germ layers: the ectoderm, the mesoderm and the endoderm of the hindgut rudiment. It extends anteriorly in a graded pattern in the neural tube, notochord and paraxial mesoderm, but shows a sharp boundary in the endoderm at the junction between the foregut and midgut. From midgestation onwards, *Cdx2* disappears in ectodermal and mesodermal derivatives, but remains selectively expressed throughout adult life in the midgut and hindgut endoderm forming the small intestine and colon epithelium[3]. Distinct regions of the *Cdx2* promoter cooperate to generate this complex expression pattern[4].

Functional studies have revealed the lethality of homozygous *Cdx2*$^{-/-}$ knockout mutants as early as day 3.5 *pc*, which demonstrates the primordial role of this homeobox gene in the trophectoderm and its extra-embryonic derivatives[5-7]. Then during early development of the embryo, *Cdx2* participates in the posterior elongation and patterning of the body[8,9]. Further studies to determine the precise function(s) of *Cdx2* in the gut at later embryonic stages and postnatally were precluded by the very early lethality of *Cdx2*$^{-/-}$ mutants, which therefore needed developing a conditional knockout approach. These studies have highlighted the key role played by this homeobox gene in the digestive tube. Indeed, the endoderm-specific ablation of *Cdx2* shortly after gastrulation or at midgestation results in embryonic/perinatal death associated to posterior gut truncations and to the transformation of the intestinal endoderm into esophageal-like or gastric-like epithelium; thus, *Cdx2* is a key actor of gut morphogenesis and the one gene that determines the tissue-identity of the presumptive intestinal endoderm[10,11]. This conclusion drawn from animal models is corroborated by observations made in humans that show a close relationship between the presence or inversely the absence of *Cdx2* respectively in intestinal-type Meckel's diverticulum and in congenital gastric-type metaplasia of the gut[12]. Beyond embryonic development, the continued expression of *Cdx2* is required throughout life in the continuously-renewing intestinal epithelium. Indeed, conditional invalidation in the adult gut epithelium leads to death, as a result of defects in cell renewal and in digestive functions, linked to a shift from intestinal-type to gastric-type differentiation[13,14]. The function of *Cdx2* is relevant already in stem cells, which is underlined by the inability of knockout Crypt Base Columnar cells (CBC, Lgr5+ cells) to produce typical "mini-intestine" organoids *in vitro*, unlike wild type CBC cells[14]. Conversely, *Cdx2* overexpression in the gut is also lethal because of an excessive epithelial maturation[15]. Together, these results emphasize the complex role played by *Cdx2* in the adult gut epithelium: it provides the stem cells with the information needed for maintaining their intestinal identity, it participates in the cellular organization of the stem cell niche and in cell renewal, and it controls the terminal differentiation of the mature digestive cells.

In addition to its crucial role in intestinal homeostasis, *Cdx2* is important in pathologies, in particular in colon cancers. Its expression decreases and becomes heterogeneous in a significant proportion of tumors, which correlates with poor prognosis and reduced disease free survival[16-18]. Experimentally in mice, a decline of Cdx2 contributes to tumor progression[19,20] and malignant cell dissemination[21], leading to attribute a tumor sup-

pressor role to *Cdx2* in the gut. Besides, although Cdx2 is physiologically restricted to the intestinal epithelium, its expression becomes ectopically turned on outside the gut in a number of epithelial organs in pathological settings associated with chronic inflammation. This is the case for instance of the gastroesophageal reflux in the esophagus and of *Helicobacter pylori* infection in the stomach[22]. The ectopic expression correlates with the development of intestinal-type metaplasias, as observed in Barrett's esophagus and in gastric intestine-type metaplasia. In the latter case, transgenic mice have demonstrated that Cdx2 is the actual driver of the abnormal intestinal transdifferentiation of the gastric mucosa[23,24]. Intestinal-type metaplasia being generally considered as a precancerous lesion, this opens the question whether Cdx2 could have a pro-oncogenic potential outside the gut. Yet, its expression tends to decrease during the pathological sequence from intestinal-type metaplasia to adenomas and carcinomas, and this decline is a factor of poor prognosis in gastric cancers[25-28], suggesting a tumor suppressor activity after the onset of the cancerous process. Thus, whether Cdx2 exerts a pro-oncogenic or a tumor suppressor activity, or both, in intestinal-type adenocarcinoma outside the gut is still under debate. Ectopic expression of *Cdx2* is also a frequent event in acute myeloid leukemia and acute lymphoblastic leukemia; in these cases, experimental evidences in mice support an oncogenic role[29-30].

## CDX2 HOMEOPROTEIN IS A TRANSCRIPTION FACTOR

Homeobox genes are characterized by a conserved 180-bp nucleotide sequence encoding the homeodomain of 60 amino-acids, rich in basic residues[31]. This domain organizes into three alpha-helixes arranged in a helix-turn-helix conformation, and constitutes the DNA-binding domain of the protein. In typical homeoproteins, this domain is preceded at the N-side by the transactivation domain and followed at the C-side by a domain of various lengths whose function is poorly documented; few homeoproteins present a second DNA-binding domain.

Following the initial discovery of a short element of the *Cdx2* gene by sequence homology search[32], the full-length protein was characterized a few years later as a transcriptional activator of the insulin and sucrase-isomaltase gene promoters[33,34]. Structure/function analyses have demonstrated its actual properties of DNA-binding transcription factor. The DNA interaction with the homeodomain was proven by Electromobility Shift and DNase protection assays[34,35], the consensus *cis*-element being determined for Caudal-type homeoproteins by SELEX [5'-(A/C)TTTAT(A/G)-3'][36] and later refined for Cdx2 by ChIP [5'-(A/C)N(A/T)N(T/A/G)(T/C)(T/A)(T/A)A(T/C)(T/G/A)(G/A)(C/T)(C/A/T)-3'][37]. The transcriptional activity of the domain located upstream of the homeodomain was established using chimeric and mutant proteins[38,39]. This transactivation domain contains phosphorylation sites that positively or negatively regulate its activity, depending on the residues that is phosphorylated[40,41]. In addition, the region of Cdx2 located downstream of the homeodomain also contains a cluster of phosphorylation sites that constitutes a signal for ubiquitin- and proteasome-dependent degradation of the protein, and therefore that regulates its turnover[42].

In addition to sucrase-isomaltase, the Cdx2 homeoprotein regulates the promoter activity of a number of genes involved in various intestinal functions like digestive hydrolases and transporters (lactase, hephaestin, ASBT)[43-45], mucins (Muc2, Muc4)[46,47], cell adhesion molecules (Cadherin-17, Claudin-2, Mucdhl)[48-50], receptors and signaling molecules[51-53], regulatory peptides (proglucagon)[54] and others. This gives Cdx2 a central role in both absorptive and secretory lineages in the gut. More recently, a comprehensive study by ChIP-seq has revealed that Cdx2 dynamically interacts with nearly 17000 chromatin binding sites across the whole genome, about 700 being specific of proliferating cells and about 14000 of differentiated cells[37]. In both cellular states, Cdx2 occupancy correlates with selective enhancer elements placed in an active chromatin conformation. Interestingly, those enhancers bound to Cdx2 in differentiated cells are enriched in sites co-occupied by HNF4, whereas enhancers bound to Cdx2 in proliferating cells are enriched in sites co-occupied by GATA6 and also by Tcf4, a crucial mediator of Wnt signaling required for stem cells maintenance in the gut[37,55]. Thus, the co-occupancy of chromatin enhancers by Cdx2 in combination with various transcription factors provides the rationale for understanding the transcriptional bases of the stepwise functions of this homeoprotein during the constant renewal of the intestinal epithelium, in stem cells, proliferating progenitors and mature differentiated cells. Altogether, these data establish at the molecular level the role of Cdx2 as a DNA-binding transcription factor central for gut homeostasis.

## NONTRANSCRIPTIONAL ACTIVITIES OF THE CDX2 HOMEOPROTEIN

Although the Cdx2 protein is primarily a transcription factor, recent data have revealed novel mechanisms of action that are nontranscriptional. Compared to the DNA-binding-dependent transcriptional activity of Cdx2, we define below nontranscriptional activities

as activities being independent of DNA-binding or independent of transactivation.

### Cell proliferation

In several human colon cancer cell lines, increasing the level of Cdx2 reduces cell proliferation[21,56,57], which is at least in part due to the transcriptional activation of the promoter of the gene encoding the cyclin-dependent kinase inhibitor $p21^{WAF}$[58]. Based on the observation that the hemizygous reduction of *Cdx2* in mice increases intestinal cell proliferation and also the susceptibility to colon cancer in $Apc^{+/716}$ mutants[20], Aoki *et al*[59] recently displayed a novel mechanism of cell cycling inhibition by Cdx2, independent of its transcriptional activity. Indeed, cell cycling inhibition is achieved as efficiently by the wild type protein Cdx2 and by mutants unable to bind the DNA. Moreover, mutants lacking the transactivation domain, although less efficient than the wild type protein, also reduce cell proliferation. The mechanism of inhibition is still not fully elucidated. However, evidence is provided that it results from the mRNA-independent increase of the level of cyclin-dependent kinase inhibitor $p27^{Kip1}$ linked to its stabilization by blocking ubiquitination and degradation by the proteasome.

The Cyclin-dependent kinases and their inhibitors involved in the correct progression through the cell cycle are controlled by several signaling pathways that converge in stem cells and progenitors to fuel the constant renewal of the intestinal epithelium. The canonical Wnt pathway is a major player of this process that regulates downstream targets like the CyclinD1 and c-myc promoters *via* the formation of the bipartite transcriptional complex made of β-catenin bound to Tcf4. In addition to its role in intestinal homeostasis, this pathway is also a leading actor of colon tumorigenesis when over-activated. Genetic and functional interactions between the Wnt pathway and *Cdx2* are multiple and far from being fully elucidated. In this context, Guo *et al*[60] have reported that Cdx2 can interfere with this pathway in colon cancer cells to lessen cell proliferation. Mechanistically, the Cdx2 protein interacts with β-catenin, thus preventing the latter to recognize the DNA-binding factor Tcf4 and activate the pathway. The interaction between Cdx2 and β-catenin requires the region of the homeoprotein preceding the homeodomain but uses elements separate from those needed for transactivation; indeed, mutants in this domain deficient in transcriptional activity can still bind β-catenin and prevent its interaction with Tcf4. Therefore, these data illustrate a novel transcription-independent mechanism of action of Cdx2 whereby the homeoprotein interacts with and blocks the recruitment of a transcriptional activator on the chromatin.

A similar relationship has been described between Cdx2 and another signaling pathway, NF-κB, also relevant in the field of colon cancer. Beside the intrinsic oncogenic mutations affecting cell proliferation and apoptosis, the abnormal growth and invasive properties of tumor cells also result from altered interactions with their microenvironment. Prostaglandins, especially the E2 series (PGE2) synthesized from arachidonic acid by Cyclooxygenase-2 (Cox-2), accumulate in the microenvironment of the tumor cells and stimulate their proliferation and invasion potential. The source of PGE2 is multiple, including the malignant cells themselves. This is linked to the up-regulation of Cox-2 in tumor cells in response to several signaling pathways, among which the NF-κB cascade. Interestingly, Kim and coworkers[61], and other later[62], have reported that Cdx2 inhibits *Cox-2* expression and consequently the production of PGE2, in spite of the absence of *cis*-element for the binding of Cdx2 in the *Cox-2* gene promoter. Instead, the Cdx2 homeoprotein achieves its effect by interacting with the p65 subunit of NF-κB and preventing its binding to the corresponding DNA elements of the *Cox-2* promoter. Thus, in the case of NF-κB as shown above for the Wnt pathway, Cdx2 intercepts signaling mediators when they reach the nucleus to divert them from their chromatin targets and therefore interrupt the signaling cascade.

### DNA repair

The *Cdx2* gene exerts a tumor suppressor activity at its physiological site of expression, the gut, but it expected to be pro-oncogenic when ectopically expressed in the hematopoietic lineage. In order to get an enlightenment of the molecular bases of these opposite functions, Renouf *et al*[63] have hypothesized that the ultimate output of Cdx2 could depend on the cellular context in a given tissue or in other words on specific partners interacting with the homeoprotein. Comparative proteomics identified the KU70/80 complex as a partner of Cdx2 selectively in colon cancer cells in contrast to leukemia cells, although KU70/80 is widely expressed in both cell types. KU70/80 is involved in a large panel of nuclear functions among which the recognition of double-strand DNA breaks (DSB) to initiate the complex molecular process leading to DNA repair. Functional studies have revealed that Cdx2, through its interaction with KU70/80, inhibits DSB DNA repair only in colon cancer cells but not in leukemia cells, and hence compromises colon cancer cell survival after a genotoxic stress. This inhibitory effect is associated to a reduction of the kinase activity of DNA-PKcs recruited into the repair complex by KU70/80 at the site of DNA break. Noteworthy, Cdx2 exerts its inhibitory effect independently of its transcriptional activity since a mutant devoid of transactivation domain is as efficient as the full-length homeoprotein. This non-

transcriptional function of Cdx2 on DSB DNA repair is particularly relevant from the perspective of its tumor suppressor role in the gut, especially with regard to the resistance to γ-irradiation-induced apoptosis[19] and to the chromosomal instability[20] observed when the Cdx2 level is decreased in the colon epithelium of *Cdx2*$^{+/-}$ mice.

### *Pre-messenger RNA splicing*

In a recent study conducted by Witek *et al*[64], a novel mRNA isoform expected to result from the alternative splicing of the *Cdx2* pre-messenger RNA has been described. Alternative splicing uses a non-conventional splicing donor site at the 5'-extremity of the second Intron which, in combination with the acceptor splicing site of this Intron, creates a 4-bases deletion compared to the classical *Cdx2* mRNA and hence a frame-shift. The resulting protein variant, Cdx2-AS, shares with Cdx2 the transactivation domain, the first two alpha-helixes of the homeodomain but the 85-amino-acids region corresponding to the third alpha-helix and C-side domain of Cdx2 are replaced in Cdx2-AS by a 42-amino-acid domain unrelated in sequence to the C-end of Cdx2. The absence of the third alpha-helix, crucial for the interaction of the homeodomain with the major groove of DNA[62], makes the Cdx2-AS variant a DNA-binding-inefficient protein. Consequently, this variant fails to transcriptionally activate typical promoter targets of Cdx2. Nonetheless, the new 42-amino-acids domain at the C-end of Cdx2-AS is rich in Serine and Arginine residues and presents some degree of sequence similarity with SR-proteins involved in RNA splicing. Functional studies established that it actually modulates the splicing pattern of genes expressed in gut cells. Therefore, these data point out the existence of an original splicing variant of *Cdx2* encoding a transcription-deficient nuclear factor that acts instead as a regulator of another major nuclear functions: the processing of pre-messenger RNAs.

## OPEN QUESTIONS

The data summarized above strongly support that Cdx2 exerts transcription-independent functions, as already shown for other transcription factors as important as p53, c-Myc and E2F1[65-67], as well as for few homeoproteins of the Hox family[68]. Most of the examples illustrating here the transcription-independent functions of Cdx2 are related to clinical situations, especially cancer. In particular, the inhibitory effect of Cdx2 on Wnt signaling has been studied in the context of a constitutively activated (oncogenic) form of β-catenin[60], while the effect on DSB DNA repair was evaluated in the perspective of the treatment of cancer cells with potential genotoxic drugs. It is thus important, for the future, to address whether the balance between the transcriptional and nontranscripitonal functions of Cdx2 is stable or if it changes in clinical situations (colon cancer, inflammatory bowel diseases, metaplasia related to the ectopic expression of *Cdx2*, leukemia) compared to the physiological situation, or in other words if the nontranscriptional functions of Cdx2 become particularly relevant in diseases and/or in response to therapeutic treatments. In addition, it would also be interesting to decipher and compare the respective roles of the transcription-independent *vs* -dependent functions of Cdx2 in the sequential cellular states characterizing the constant renewal of the intestinal epithelium, in stem cells, committed progenitors and postmitotic differentiated cells. These issues can also be raised for the developmental roles of *Cdx2*, in the presumptive gut, in body axis elongation and patterning, and even in extraembryonic tissues.

The nontranscriptional functions of Cdx2 are largely related to the capacity of the homeoprotein to interact with a variety of partners. Proteomics technology has substantially improved during the last years allowing investigating protein-protein interactions at a large scale; however no comprehensive study has been performed so far to characterize the interactome of Cdx2. This objective is challenging because such studies can be complicated by the fact that molecular interactions are strongly reliant on the cellular context. The context is defined on the one hand by the panel of putative Cdx2 partners expressed within a given cell, and on the other hand by the posttranslational modifications of Cdx2 and its partners that condition the physical interactions. Therefore, characterizing the transcription-independent activities of Cdx2 not only requires identifying its multiple interacting proteins but also determining the pattern of posttranslational modifications. The Cdx2 protein has already been shown to be ubiquitinylated and phosphorylated[40-42], the phosphorylation at different sites producing different effects. Additional types of modifications are also possible like acetylation, methylation, PARylation and others. Conversely, based on similar considerations, another stimulating issue is to know if Cdx2, when it interacts with a given partner, modulates all the functions attributed to this partner or only a subset of these functions. For instance, the KU70/80 complex has many roles beyond DSB DNA repair, including the regulation of gene expression, the preservation of telomeres and the control of apoptosis. It is not known yet if Cdx2, through KU70/80, may also belong to and affect the molecular complexes containing proteins involved in these functions, like hTERT and Bax.

Molecular interactions between partners are highly dependent on the compartimentalization of proteins. This issue is particularly relevant to connect post-translational modifications with diseases. Interestingly, whereas the Cdx2 protein is restricted to the nucleus in normal intestinal cells, a diffuse

cytoplasmic immunostaining often appears in histological sections of human colorectal tumors and already in a number of adenomas, suggesting that a fraction of the homeoprotein is abnormally redirected outside its physiological compartment. This could be related to an unusual form of posttranslational modification and was generally considered as a sign of inactivity because the transcription factor is remote from the chromatin. However, since Cdx2 also has transcription-independent functions, one can now speculate that the homeoprotein meets new partners in the odd location and therefore affects a subset of cytoplasmic functions specifically in disease cells, that cannot be anticipated from studies conducted in healthy cells. Investigating this issue is important but challenging because, to our knowledge, no human colon cancer cell line has been reported so far to recapitulate the diffuse distribution of Cdx2 in the cytoplasm; without appropriate model, this makes it difficult to mechanistically approach the role of cytoplasmic Cdx2 in malignant cells.

## CONCLUSION

In conclusion, beside it's primarily role of transcription factor, more and more data support the emerging concept that the Cdx2 homeoprotein also exerts transcription-independent activities. These activities considerably broaden the field and the mechanisms of action of this factor, being now involved in new and important cellular and molecular functions, like DNA repair and RNA splicing. These functions were not previously anticipated from the transcriptomic data related to Cdx2. Determining the complete list of functions regulated by Cdx2 using a nontranscriptional mechanism, together with the list of Cdx2 partners for these functions, is a very ambitious objective at the molecular and cellular levels, and it is even more challenging to put these nontranscriptional functions into a physiological or pathological context with appropriate genetic models in mice. These objectives stimulate new directions of research, the results of which should extend our knowledge on the development and homeostasis of the gut as well as on malignant and/or inflammatory diseases.

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