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Running title: Homeobox genes differently control DSB DNA repair

ABSTRACT

Homeobox genes, involved in embryonic development and tissues homeostasis in adults, are often deregulated in cancer, but their relevance in pathology is far from being fully elucidated. In colon cancers, we report that the homeoproteins HoxB7 and Cdx2 exhibit different heterogeneous patterns, Cdx2 being localized in moderately altered neoplasic glands in contrast to HoxB7 which predominates in poorly-differentiated areas; they are coexpressed in few cancer cells. In human colon cancer cells, both homeoproteins interact with the DNA repair factor KU70/80, but functional studies reveal opposite effects: HoxB7 stimulates DNA repair and cell survival upon etoposide treatment, whereas Cdx2 inhibits both processes. The stimulatory effect of HoxB7 on DNA repair requires the transactivation domain linked to the homeodomain involved in the interaction with KU70/80, whereas the transactivation domain of Cdx2 is dispensable for its inhibitory function, which instead needs the homeodomain to interact with KU70/80 and the C-terminal domain. Thus, HoxB7 and Cdx2 respectively use transcription-dependent and -independent mechanisms to stimulate and inhibit DNA repair. In addition, in cells co-expressing both homeoproteins, Cdx2 lessens DNA repair activity through a novel mechanism of inhibition of the transcriptional function of HoxB7, whereby Cdx2 forms a molecular complex with HoxB7 and prevents it to recognize its target in the chromatin. These results point out the complex interplay between the DSB DNA repair activity and the homeoproteins HoxB7 and Cdx2 in colon cancer cells, making of the balance between these factors a determinant and a potential indicator of the efficacy of genotoxic drugs.

Keywords: homeobox gene, transcription, nontranscriptional, KU complex

1. INTRODUCTION

Transcription factors encoded by homeobox genes are major determinants of embryonic development, whose mutations generate congenital morphological defects. Several of these genes remain expressed throughout adulthood to control tissue homeostasis, while being deregulated in pathological settings including cancers (Samuel and Naora, 2005; Shah and Sukumar, 2010). Their role in cancer is widespread: they can act as tumor suppressors, or as oncogenes, and several homeogenes even exhibit both anti-oncogenic and pro-oncogenic activities (Yamaguchi et al., 2013).

The Hox-B cluster homeobox gene 7, HoxB7, participates in embryonic patterning and axis formation, and is abnormally overexpressed in many cancers including myeloma, breast, lung, pancreas, ovarian, oral and colon cancers. Overexpression is associated with poor prognosis, cell proliferation (De Souza Setubal Destro et al., 2010; Liao et al., 2011), epithelial to mesenchymal transition (Wu et al., 2006), tumor-associated angiogenic switch (Carè et al., 2001) and metastasis (Chen et al., 2008; Yuan et al., 2014). The paraHox cluster homeobox gene Cdx2 also has multiple developmental functions, and it remains active throughout adulthood selectively in the gut to determine and regulate intestinal identity and homeostasis (Gao et al., 2009; Stringer et al., 2012). However in contrast to HoxB7, its expression is frequently decreased and heterogeneous in colon cancer, which correlates with poor prognosis (Baba et al., 2009; Brabletz et al., 2004; De Sousa E Melo et al., 2013) and contributes to tumor progression (Aoki et al., 2003; Bonhomme et al., 2003) and malignant cell dissemination (Gross et al., 2008). Thus, HoxB7 has a general oncogenic potential, unlike Cdx2 which is considered as a gut tumor suppressor. Recently, both HoxB7 and Cdx2 have been shown to control double-strand break (DSB) DNA repair activity, yet in opposite ways. Indeed, in breast cancer cells, the oncogenic potential of HoxB7 has been linked to its ability to stimulate DSB DNA repair by interacting with the KU70/80 complex (Rubin et al., 2007). Conversely, in the colon epithelium, the increased chromosomal instability and the resistance to γ-irradiation-induced apoptosis observed upon reducing Cdx2 levels (Aoki et al., 2003; Bonhomme et al., 2003) is paralleled by an inhibitory effect of this homeoprotein on DSB DNA repair, also mediated by its interaction with the KU70/80 heterodimer (Renouf et al., 2012).

While the opposite effects of HoxB7 and Cdx2 have been reported so far in tumors of different origin, the aim of the current study was to establish the DNA repair properties of both homeoproteins in a single tumor type, the colon cancer, in order to compare their

mechanism of action on DNA repair and to explore possible functional interactions between both homeoproteins.

MATERIAL and METHODS

2.1 Mice, cell lines and transfections, Plasmids, Antibodies

Mouse treatment, cell culture protocols and material used in this study are described in supplementary informations.

2.2 Cell survival assays

After 24 hours of transfection, HCT116 cells were treated for 24 hours with 20µM of etoposide (Sigma Life Science) in the culture medium. They were then trypsinized and 10^5 cells were seeded in culture medium and grown for 2 weeks. Cell colonies were fixed with 70% ethanol, stained with 0.1% crystal violet and counted using ImageJ software (W Rasband, Bethesda, MA).

2.3 Double-Strand Break (DSB) DNA repair assays

For the in vivo assays, pGL3-control plasmid was cut by HindIII and purified using Qiagen PCR purification kit (Qiagen, Hilden, Germany). HCT116 cells (1.5×10^6) were co-transfected with 250 ng of cut pGl3-control, with various amounts of plasmids encoding F-Cdx2, F-HoxB7 or mutated forms of these homeoproteins and with pRL-null as an internal control (Promega, Madison, WI). Forty-eight hours after transfection, Firefly and Renilla luciferase activities were measured using the dual reporter luciferase assay (Promega, Madison, WI) and a Lumistar luminometer (BMG, Offenbourg, Germany).

For the in vitro assays, nuclear extracts were prepared as described previously (Renouf et al., 2012). End-joining reactions were performed during 1 hour at 25°C using 250 ng of plasmid pRL-null previously cut with SalI and 20 µg of nuclear extracts in a final volume of 20 µL containing 20 mM Hepes-KOH pH 7.4, 10 mM MgCl2, 80 mM KCl, 1 mM ATP, 1 mM DTT and protease inhibitor cocktail (Roche, Mannheim, Germany). The reaction was stopped by adding 0.5% SDS, 50 mM EDTA and 1µg/µL of proteinase K and incubated for 1 hour at 37°C. DNA was purified by phenol / chloroform extraction and separated by electrophoresis on a 0.7 % agarose gel at 2 V/cm. DNA bands were visualized using Ethidium Bromide and UV illumination and quantified by the ImageJ software.

2.4 Immunoprecipitation and Western blots

Forty-eight hours after transfection, cells were lysed 10 min in lysis buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (Roche, Mannheim, Germany). The lysate was centrifuged for 10 min at 20,000 g at 4°C. Immunoprecipitation was performed on 1 mg of protein with 30 µL anti-Flag beads (Anti-Flag M2 Affinity Gel, Sigma Aldrich) or anti-HA beads (Anti-HA Affinity Matrix, Roche), for 2 hours at 4°C in 1 mL of lysis buffer. Immunoprecipitates were washed 3 times in lysis buffer and eluted with SDS–PAGE sample loading buffer (Biorad, Hercules, CA).

When indicated, HCT116 cell extracts (1 mg) were treated for 30 min with 400 µg/mL Ethidium Bromide prior to the addition of the beads. Cell extracts or immunoprecipitates were analyzed by SDS-PAGE (12%), transferred to nitrocellulose filters and analyzed by Western blot and ECL detection (GE Company, Fairfield, CT).

2.5 Luciferase assays

HCT116 cells (1.5 x 10^6) were co-transfected with the reporter plasmids pTCBS or CDH17-Luc (250 ng), with the plasmids encoding F-HoxB7, F-Cdx2 or mutated forms, and with pRL-null as internal control (Promega, Madison, WI, USA). Firefly and Renilla luciferase activities were measured 48 hours after transfection using a dual reporter luciferase assay (Promega) with a Lumistar luminometer (BMG, Offenbourg, Germany).

2.6 Chromatin ImmunoPrecipitation (ChIP)

Chromatin Immunoprecipitation was performed using Magna ChIp G kit (Millipore, Darmstadt, Germany) following the instructions of the supplier with few modifications. HCT116 cells (10^6 for each ChIP experiment) were co-transfected with the plasmids pF-HoxB7 and/or pHA-Cdx2 together with pTCBS. After cell disruption in 50 µL of Cell Lysis Buffer and sonication using a Bioruptor (Diagenode, Seraing, Belgium) for 12 min, chromatin immunoprecipitation was performed on 1 mg of whole protein extract using anti-Flag antibody (2µg of M2 affinity purified antibody (Sigma Aldrich) and G magnetic beads or with beads coupled to anti-HA antibody (Anti-HA Affinity Matrix, Roche, Mannheim, Germany) overnight at 4°C. Co-immunoprecipitated DNA was purified using Qiagen PCR purification kit (Qiagen, Hilden, Germany) and eluted in 35 µL of EB buffer. Purified DNA (2 µL) were used for quantitative real time PCR with FastStart Sybr Green Master (Roche) and primers flanking the HoxB7 binding sites in pTCBS (CTCAGATCCAAGCTTGTCGA / TTCGAATTCGCCAATGACAAGA).

2.7 In vitro poly-ADP-ribosylation (PARylation) assay

HCT116 cells transfected for 48 h with plasmid encoding the Flag-tagged protein to be checked, were treated with 100 nM of PARP inhibitor (Ku0058684) (Farmer et al., 2005) for 2 h to avoid any in vivo PARylation of the the neosynthetized protein and then lysed in lysis buffer (50 mM Tris pH7.4, 500 mM NaCl, 1mM EDTA, 1% Triton X100, 100nM PARP inhibitor, protease inhibitor (Roche, Mannheim, Germany)). Total protein extracts (1mg) was used to immunoprecipitate Flag-Cdx2 with Flag beads (M2 affinity purified, Sigma Aldrich) for 2 h in lysis buffer. Beads were washed twice in wash buffer (50mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 0.2 % NP40, 100 nM PARP inhibitor) and twice in buffer A (50 mM Tris pH7.4, 0,2 mM DTT, 4 mM $MgCl_2$, 0,1 µg/µl BSA). Beads were suspended in 50 µL of buffer A containing 1 µg of purified recombinant PARP1. In parallel, 50 µL of buffer A were mixed with 20 ng/µL of fragmented DNA, 0.2 µM of NAD and 1µl of ^{32}P-NAD. These two solutions were mixed and incubated 25 min at 25°C. After 4 washes with dilution buffer containing 20 mM Tris pH7.5, 400 mM NaCl, 0,5 mM protease inhibitor (Roche, Mannheim, Germany) and 0,1% NP40, proteins were eluted with SDS–PAGE sample loading buffer (Biorad, Hercules, CA), separated by SDS-PAGE, transferred to nitrocellulose filters and revealed by autoradiography.

2. RESULTS

3.1 Expression profiles of HoxB7 and Cdx2 in the gut and in colon tumors

In a first attempt, the expression patterns of the Cdx2 and HoxB7 homeoproteins were compared in the mouse intestinal epithelium and in colon tumors induced by the chemical carcinogen, Azoxymethane. In the normal gut, Cdx2 was detected in the differentiated cells of the villi as well as in cells of the proliferating compartment, the crypts, whereas HoxB7 was only expressed in crypt cells (Figure 1A). In colon tumors, Cdx2 became heterogeneous and barely detectable in strongly altered neoplasic glands. Inversely, HoxB7 immunostaining increased, especially in poorly-differentiated area of the tumors (Figures 1B and 1C). Few neoplasic glands and/or cells within neoplasic glands co-expressed Cdx2 and HoxB7, as assessed by double immunofluorescence staining (Figure 1C). As in the tumors, the analysis of human colon cancer cell lines revealed some of them predominantly expressing Cdx2 (Caco2TC7), others HoxB7 (HCT116, HT29), while others co-express both homeoproteins (SW480, DLD1) (Figure 2A).

3.2 Opposite effects of HoxB7 and Cdx2 on DSB DNA repair in colon cancer cells

In colon cancers, HoxB7 activates cell proliferation (Liao et al., 2011). Whether it also modulates DSB DNA repair remains unknown. To address this issue, we analyzed DSB DNA repair activity in colon cancer cells overexpressing Flag-HoxB7 and compared it to cells expressing Flag only as control, or Flag-Cdx2. DSB DNA repair activity was determined using an in vitro assay that measures the capacity of nuclear extracts to perform end-joining of a linearized plasmid giving rise to multimers, and an in vivo assay in which the pGl3-control reporter plasmid was cut between the SV40 promoter and the luciferase-encoding sequence prior to transfection, so that luciferase activity is produced only when the plasmid is repaired within cells. The results shown in Figures 2B and 2C demonstrate a stimulatory effect exerted by HoxB7 in colon cancer cells HCT116, both in vitro and in vivo. In contrast to HoxB7, and corroborating our previous observations (Renouf et al., 2012), both assays showed an inhibitory effect of Cdx2 on DSB DNA repair activity in these colon cancer cells (Figure 2B and 2C). Similar opposite effects were observed in in vivo DSB DNA repair assays using two other colon cancer cell lines: the DLD1 (Figure 2C) and HT29 cells (not shown). In mammary

cancer cells, the stimulatory effect of HoxB7 on DSB DNA repair is linked to its interaction with the KU70/80 complex (Rubin et al., 2007). Using co-immunoprecipitation experiments, we detected a similar HoxB7-KU70/80 association in colon cancer cells, indicating that HoxB7 also interacts with KU70/80 in gut tumors (Figure 2D). This was also shown for Cdx2 interacting with KU70/80 (Figure 2D). Together, these results provide evidence that HoxB7 and Cdx2 exert opposite effects on DSB DNA repair in colon cancer cells, being respectively stimulator for HoxB7 and inhibitor for Cdx2, while both homeoproteins share a common partner, the KU70/80 complex.

3.3 Relevance of HoxB7 and Cdx2 on cell survival after a genotoxic stress

The relevance of the opposite effects of HoxB7 and Cdx2 on DSB DNA repair was investigated in colon cancer cells subjected to a genotoxic stress. For this purpose, HCT116 cells were transfected with the plasmids encoding Flag-tagged HoxB7 or Cdx2, treated with the topoisomerase II inhibitor etoposide to induce double-strand breaks and allowed to recover for 2 weeks. As illustrated in Figure 2E, the overexpression of HoxB7 stimulated the survival of etoposide-treated cells, whereas Cdx2 reduced their survival. Therefore, there is a strong parallel between the respective effects of HoxB7 and Cdx2 on DBS DNA repair activity and their impact on colon cancer cell survival after a genotoxic stress.

3.4 Distinct domains involved in the opposite functions of HoxB7 and Cdx2

To explore the molecular mechanism(s) related to the opposite effects of Cdx2 and HoxB7 on DSB DNA repair, we constructed plasmids encoding truncated forms of either homeoproteins. They allowed studying the effect of these truncated mutants on DNA repair activity using in vitro and in vivo assays (Figures 3A and 3C), as well as addressing their capacity to interact with the KU70/80 complex by immunoprecipitation (Figures 3B and 3D). Every mutant contained the Nuclear Localization Sequence. It has already been reported that the transactivation domain of the Cdx2 protein corresponds to the N-half of the protein, located upstream of the DNA-binding homeodomain (Rings et al., 2001). The truncated protein overlapping the homeodomain and C-terminal region of Cdx2 but devoid of N-terminal transactivation domain (F-C2^{HD+Ct}) was as efficient as full-length Cdx2 to inhibit DNA repair and it also retained the capacity to interact with KU70/80. In contrast, the mutant containing the N-terminal transactivation domain and the first two alpha-helixes of the

homeodomain (F-C2^{Nt}) failed to inhibit DNA repair as well as to interact with the KU complex. Interestingly, the mutant limited to the homeodomain alone (F-C2^{HD}) could no longer inhibit DNA repair although it was still able to interact with KU7/80. Altogether, these results indicate that the N-terminal domain of Cdx2, responsible for its transcriptional activity, is dispensable for DSB DNA repair inhibition, and that this inhibitory function requires the interaction of the homeodomain with KU70/80 through the third alpha helix, plus the C-terminal domain. Similar truncated mutants were constructed for HoxB7, leading to very different results (Figures 3C, 3D). In particular, the absence of N-terminal transactivation domain in HoxB7^{HD+Ct} abrogated the stimulatory effect of DSB DNA repair activity, although the interaction with KU70/80 was preserved. In addition, even when the transactivation domain and the first two alpha-helixes of the homeodomain were present in F-B7^{Nt}, this truncated mutant was unable to stimulate DSB DNA repair and it also failed to interact with KU70/80. Finally, the mutant F-B7^{HD} corresponding to the homeodomain alone still associated with the KU complex but did not stimulate DNA repair. Thus, to stimulate DSB DNA repair activity, HoxB7 requires the intact DNA-binding homeodomain to interact with KU70/80 and the transactivation domain. Based on these results, we investigated if KU70/80 is a regulator of the transcriptional activity of HoxB7. Using a reporter vector with multimerized consensus Hox binding elements, pTCBS, KU70/80 was found to stimulate the transactivation potential of HoxB7 (Figure 3E). Altogether, these results demonstrate first, that both HoxB7 and Cdx2 require the third helix of their homeodomain to interact with KU70/80 and second, that their specific and opposite effects on DSB DNA repair are depending on additional and distinct domains: the C-terminal domain of Cdx2 to inhibit DNA repair and the N-terminal transactivation domain of HoxB7 to stimulate it.

To strengthen this conclusion, we constructed chimeric proteins composed of swapped domains of Cdx2 and HoxB7 and checked their effect on DSB DNA repair with in vitro and in vivo assays (Figure 3F). The protein F-C2^{Nt}B7^{HD+Ct} in which the N-terminal transactivation domain of Cdx2 was linked to the homeodomain and C-terminal part of HoxB7, was still able to stimulate DNA repair, like HoxB7. This demonstrates that a heterologous transactivation domain, without sequence homology with the one of HoxB7, can replace it to stimulate DNA repair in the context of the HoxB7 homeodomain. Conversely, linking the homeodomain and C-terminal domain of Cdx2 to the transactivation domain of HoxB7 produced the chimera F-B7^{Nt}C2^{HD+Ct} that inhibited DNA repair to a similar extend as the full-length Cdx2 or to the mutant F-C2^{HD+Ct}; moreover, the chimera F-B7^{Nt+HD}C2^{Ct}, although not inhibitor, failed to

stimulate DSB DNA repair. Together, these data provide strong evidence that the stimulatory effect of HoxB7 on DSB DNA repair needs an active transcriptional function, whereas the inhibitory effect exerted by Cdx2 depends on its HD+Ct domains. For this inhibitory effect, the Ct domain of Cdx2 is important but requires its cognate HD for full activity.

The Ct domain of Cdx2 shown here to be crucial for DSB DNA repair inhibition contains a 4-Serine phosphorylation motif that directs polyubiquitination (Gross et al., 2005). Mutants preventing phosphorylation (HA-Cdx2$^{4S>A}$) or mimicking constitutive phosphorylation (HA-Cdx2$^{4S>E}$) still interacted with KU70/80 and were as effective as wild type Cdx2 to inhibit DSB DNA repair activity (Supplementary Figure 2), ruling out a role for the 4-Serine motif in this function of Cdx2.

Since parylation catalyzed by Poly-ADP-Ribosyl-Polymerase (PARP) is a major actor of the response to DNA damage, and since poly-ADP-ribosylation (PARylation) attenuates the transcriptional activity of HoxB7 (Robert et al., 2013; Wu et al., 2012), we checked its impact on DSB DNA repair. The PARylation of HoxB7, confirmed in vitro (Supplementary Figure 1A), reduced its transcriptional activity and also its capacity to stimulate DSB DNA repair (Supplementary Figure 1C,B), while the PARP1 inhibitor Ku0058948 had the opposite effect (Supplementary Figure 1G). This is fully consistent with a transcription-dependent effect of HoxB7 on DSB DNA repair and indicates that PARylation modulates this property. Based on these results on HoxB7, we then checked if PARylation has any effect on the inhibitory effect of DSB DNA repair by Cdx2. In vitro PARylation assays and co-immunoprecipitation experiments revealed that Cdx2 interacts with and can be PARylated by PARP1, predominantly into the HD+Ct domains (Supplementary Figure 1A, D). However, neither the overexpression of PARP1, nor its inhibition, affected the inhibitory effect of Cdx2 on DSB DNA repair; in addition, PARylation does not have any impact on its transcriptional activity assayed using the reporter plasmid CDH17-Luc (Supplementary Figure 1E-G). Thus, we identified here a novel posttranslational modification of Cdx2, PARylation, whose functional relevance remains to elucidate.

3.5 Inhibition of the transcriptional function of HoxB7 by Cdx2

Since Cdx2 and HoxB7 are co-expressed in some colon tumor cells but have opposite effects on DSB DNA repair, we next investigated the consequence of combining these homeoproteins on DNA repair. In vivo DNA repair assays showed that the inhibitory effect of

Cdx2 overpassed the stimulatory effect of HoxB7, unless the latter was in excess (Figure 4A). In line with our above results, the transcription-deficient form F-C2^{HD+Ct} also overpassed the stimulatory effect of HoxB7, in contrast to F-C2^{Nt} (Figure 4A). These data prompted us to check if Cdx2 and HoxB7 can belong to a commune protein complex. As illustrated in Figure 4B, HA-Cdx2 co-immunoprecipitated with Flag-HoxB7; the same was observed for HA-C2^{HD+Ct}. Interestingly, probing the HoxB7 / Cdx2 co-immunoprecipitates for KU70/80 also demonstrated the presence of this heterodimer (Figure 4B), which opened the possibility for the existence of a ternary complex containing KU70/80, HoxB7 and Cdx2. However, repeating the co-immunoprecipitation experiments in the presence of Ethidium Bromide led to the disruption of the associations between HoxB7 and KU70/80 and between Cdx2 and KU70/80, whereas the HoxB7-Cdx2 association was preserved (Figure 4C). Thus, the HoxB7-Cdx2 interaction does not require KU70/80 and is not mediated by DNA. Since KU70/80 is one of the most abundant proteins in a cell, we also ruled out a mechanism whereby Cdx2 would counteract the function of HoxB7 by competing for the interaction with KU70/80.

Therefore, we investigated if Cdx2 has any effect on the transcriptional activity of HoxB7, using the reporter plasmid pTCBS. As expected (Figure 4D), HoxB7 strongly stimulated pTCBS luciferase activity, in contrast to Cdx2, even at a high dose, which indicated that Cdx2 is not efficient for transactivating for Hox binding sites; however, the stimulatory effect of HoxB7 was dose-dependently reduced by Cdx2 and also by the transcription-deficient form Cdx2^{HD+Ct}. Inversely HoxB7 did not affect the transcriptional activity of Cdx2 (Figure 4E). To get insight into the mechanism by which Cdx2 counteracts the transcriptional activity of HoxB7, we performed chromatin-immunoprecipitation assays (Figure 4F). Control experiments confirmed the strong binding of HoxB7 on its cognate DNA target sites, in contrast to Cdx2. However, Cdx2 efficiently prevented the DNA binding of HoxB7. Therefore, these results highlight a new mode of functional relationship between Cdx2 and a Hox protein, based on their DNA-independent physical interaction and consequently on the inability of HoxB7 to bind the chromatin.

3. **DISCUSSION**

In this work, we highlight the differences in expression and function of the two homeodomain proteins HoxB7 and Cdx2 in the normal and cancerous intestinal epithelium. Cdx2 is expressed in both proliferating and differentiated cells of the continuously renewing

epithelium, and it becomes heterogeneous and globally reduced in colon cancers. On the contrary, HoxB7 is only expressed in the proliferating crypt cells and overexpressed in cancers. These data underline the complexity of the role played by the homeobox gene in cancer development (Shah and Sukumar, 2010). In addition, this study unveils a complex interplay between the DSB DNA repair activity and the homeoproteins HoxB7 and Cdx2 in colon cancer cells (Supplementary Figure 3). HoxB7, already reported to stimulate cell proliferation (Liao et al., 2011), also stimulates the activity of DNA repair, and consequently the capacity of malignant cells to overcome DNA breaks. This supports an oncogenic role of this homeoprotein in gut tumors. It is in clear contrast to the tumor suppressor activity attributed to the intestine-specific homeoprotein Cdx2 (Aoki et al., 2003; Bonhomme et al., 2003; Gross et al., 2008) linked to its inhibitory effect on DSB DNA repair. Importantly, although both homeoproteins exert their effects in association with KU70/80, their opposite functions rely on different molecular mechanisms, being respectively transcriptional for HoxB7 and nontranscriptional for Cdx2. Moreover, Cdx2 acts on DNA repair by two distinct ways. On the one hand, it directly inhibits the DNA repair machinery (Renouf et al., 2012). On the other hand, itcouteracts the stimulatory function of HoxB7 by interacting with it and preventing its DNA binding; a similar mechanism has been reported for Cdx2 to prevent the activation of the Cox2 promoter by NFkB (Kim et al., 2004) . Therefore, we propose that the functional interplay between Cdx2 and HoB7 evolves during the sequence from the healthy epithelium to overt cancer. Indeed, in normal cells, the response to double-strands DNA breaks depends on the actual balance between Cdx2 and HoxB7. During cancer progression, Cdx2 becomes reduced and heterogeneous, which consequently potentiates the pro-oncogenic activity of HoxB7. Hence, the results reported here suggest that the evolution of the Cdx2 / HoxB7 balance during the progression of colon cancer is functionally relevant, and that this ratio may be a determinant and an indicator of the efficacy of anticancer genotoxic drugs acting through the formation of DNA breaks.

Acknowledgements

We thank Dr V Zappavigna (Cornell University, New York, NY) and Dr S Park (John Hopkins University School of Medicine, Baltimore, MD) for providing the plasmids pTCBS and pCB6-Flag-HoxB7. This work was supported by INCa #ACM-07 (France). C.S. was

funded by the Ministère de l’Enseignement Supérieur et de la Recherche (France) and by the Fondation pour la Recherche Médicale (France).

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FIGURES LEGENDS

Figure 1 Cdx2 and HoxB7 expression in the murine gut epithelium and in colon tumors

A. Immunohistochemical staining of Cdx2 and HoxB7 proteins in crypt and villus epithelial cells of the mouse small intestine. Bar is 50 µm. B. Immunohistochemical staining of Cdx2 and HoxB7 in serial sections of two distinct regions of an AOM-induced adenocarcinoma in the mouse colon. Bar is 100 µm. C. Double-immunofluorescence detection of Cdx2 and HoxB7 in an AOM-induced colon adenocarcinoma to illustrate the various patterns of these homeoproteins. Bars are 50 µm. Colon tumors were induced in mice by intraperitoneal administration of AOM (Sigma, St Louis, MO, 10 mg/kg) once a week for five week; colon tumors were taken 25 weeks later. Immunodetection on the three samples used mouse monoclonal anti-Cdx2 antibody (Biogenex, San Ramon, CA) and rabbit anti-HoxB7 antibody (Sigma Life Science).

Figure 2 Opposite effects of Cdx2 and HoxB7 on DSB DNA repair activity and cell survival in colon cancer cells

A. Western blot analysis of the proteins Cdx2, HoxB7 and KU70/80 in human colon cancer cell lines. Actin was used as control. **B**. In vitro DSB DNA repair activity. The left panel illustrates the electrophoresis pattern of SalI-linearized plasmid pRL-null incubated in the repair buffer without nuclear extract (-), or with nuclear extracts of HCT116 cells transfected with the control vector pFlag, pF-Cdx2 or pF-HoxB7. Arrowhead: linearized plasmid; asterisk: multimers resulting from the DNA repair activity. The right panel shows the quantification of the multimers / linear ratio; the value of 1 was arbitrary attributed to nuclear extracts of pFlag-transfected HCT116 cells. Data are given as means ±SEM for 4 independent experiments; ** $p<0.01$. **C**. In vivo DSB DNA repair activity in HCT116 and DLD1 cells. Luciferase activity was measured in cells transfected with HindIII-linearized pGl3-control together with pFlag, pF-Cdx2 or pF-HoxB7. The value of 1 was arbitrary given to pFlag transfected cells. Data are given as means ±SEM for 6 independent experiments; *** $p<0.001$. **D**. Interaction between KU70/80 and the Cdx2 and HoxB7 proteins. HCT116 cells were transfected with pFlag, pF-Cdx2 or pF-HoxB7. Western blots with anti-Flag or anti-KU70 / anti-KU80 antibodies were performed on crude cell extracts (input) and after immunoprecipitation with anti-Flag antibody (IP: @Flag). **E**. Impact of Cdx2 and HoxB7 on

cell survival after etoposide treatment. HCT116 cells were transfected with pFlag, pF-Cdx2 or pF-HoxB7, treated with etoposide for 24h (20 µM, Sigma Life Science) and cultured for 2 weeks. Data are given as means of the numbers of clones ±SEM, and set to 1 for pFlag-transfected cells; * $p<0.05$.

Figure 3 Functional domains of the Cdx2 and HoxB7 proteins

A. Effect of truncated Cdx2 mutants on DSB DNA repair activity in HCT116 cells. The left panel corresponds to in vitro DNA repair assays; data are expressed as the ratio between multimers and linear DNA, and given ±SEM for 3 independent experiments; ** $p<0.01$; n.s. not significant. The right panel corresponds to in vivo DNA repair activity determined by measuring luciferase activity in cells transfected with HindIII-linearized reporter plasmid pGl3-control together with plasmids encoding the indicated truncated proteins; data are given as means ±SEM for 5 independent experiments; *** $p<0.001$; n.s. not significant. **B**. Interaction of the Cdx2 mutants with KU70/80. Co-immunoprecipitation with anti-Flag antibody in HCT116 cells transfected with plasmids encoding the indicated Flag-tagged Cdx2 truncated proteins, followed by western blots with anti-Flag and anti-KU70 / anti-KU80 antibodies (inputs were omitted for clarity). **C**. Effect of HoxB7 truncated mutations on DSB DNA repair activity. The results are expressed as in (A). Left panel: in vitro DNA repair assays, right panel: in vivo DNA repair assays. **D**. Interaction of the HoxB7 mutants with KU70/80. Co-immunoprecipitation with anti-Flag antibody in HCT116 cells transfected with the plasmids encoding Flag-tagged forms of HoxB7 truncated proteins, followed by western blots with anti-Flag and anti-KU70 / anti-KU80 antibodies (inputs were omitted for clarity). **E**. Transcription activity measured using the pTCBS reporter in HCT116 cells transfected with pFlag, pF-HoxB7 or together pF-HoxB7, pKU70 and pKU80 (F-HoxB7+KU70/80). Data are given ±SEM for 3 experiments; *** $p<0.001$. **F**. DSB DNA repair activity of chimeric proteins containing swapped domains of Cdx2 and HoxB7. The results are expressed as in (A). Left panel: in vitro DNA repair assays; right panel: in vivo DNA repair assays.

Figure 4 Interference between HoxB7 and Cdx2 for DSB DNA repair activity

A. In vivo DSB DNA repair activity was determined by measuring luciferase activity in HCT116 cells transfected with HindIII-linearized reporter plasmid pGl3-control together with the indicated amount of plasmids (ng) encoding F-HoxB7 and F-Cdx2 or truncated forms of Cdx2. The value of 1 was arbitrary given to control pFlag transfected cells. Data are given as means ±SEM for 4 independent experiments; ** $p<0.01$; n.s. not significant. **B**. Co-immunoprecipitation with anti-Flag antibody of cell extracts of HCT116 cells transfected with pFlag or pF-HoxB7 together with the plasmids encoding HA-tagged Cdx2 (HA-Cdx2) or $C2^{HD+Ct}$ (HA-$C2^{HD+Ct}$); western blots were performed with anti-Flag, anti-HA and anti-KU70 / anti-KU80 antibodies (inputs were omitted for clarity). **C**. Stability of the protein complexes in the presence of ethidium bromide (EB). Cells were transfected with pHA-Cdx2 and proteins were coimmunoprecipitated with anti-HA antibody, or they were transfected with pFlag or pF-HoxB7 and proteins were coimmunoprecipitated with anti-Flag antibodies. Co-immunoprecipitations were performed in the absence or presence of EB. Western blots used anti-Flag, anti-HA and anti-KU70 / anti-KU80 antibodies (inputs were omitted for clarity). **D**. Effect of Cdx2 on the transcriptional activity of HoxB7. Cells were transfected with the reporter plasmid pTCBS together with the indicated amounts (ng) of plasmids pF-HoxB7, pF-Cdx2 or pF-$C2^{HD+Ct}$. Luciferase data are given as means ±SEM for 3 independent experiments; ** $p<0.01$. **E**. Effect of HoxB7 on the transcriptional activity of Cdx2. Cells were transfected with the reporter plasmid pCDH17-Luc together with the indicated amounts (ng) of plasmids pHA-Cdx2 and pF-HoxB7; n.s. not significant. **F**. Chromatin immunoprecipitation was performed using anti-Flag antibody (all samples except for HA-Cdx2 immunoprecipitated with anti-HA antibody) (grey boxes) or control IgG (white boxes) in HCT116 cells transfected with the plasmids pFlag, pF-HoxB7 and/or pF-Cdx2. The results of qPCR are expressed as percent of input.

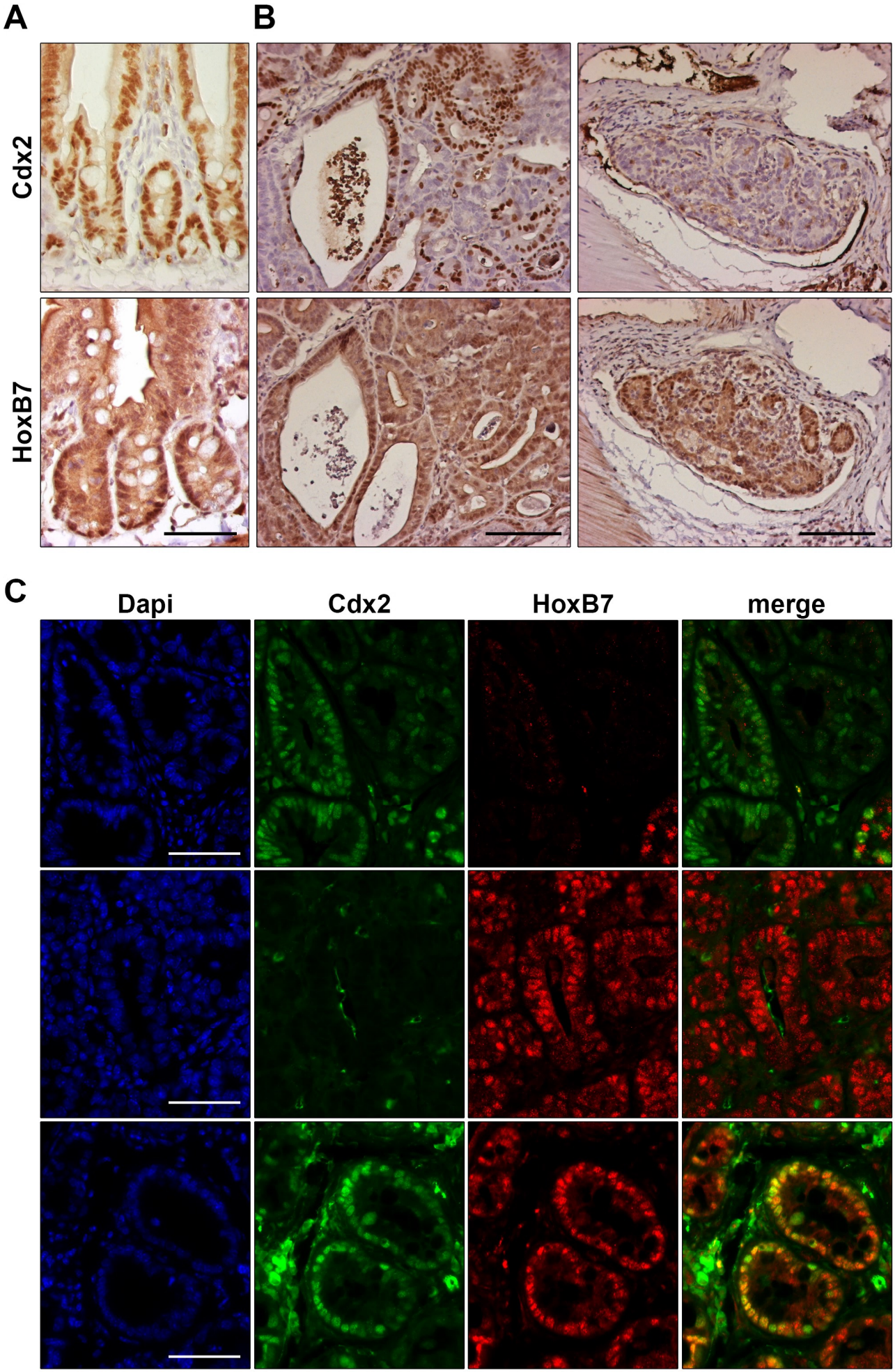
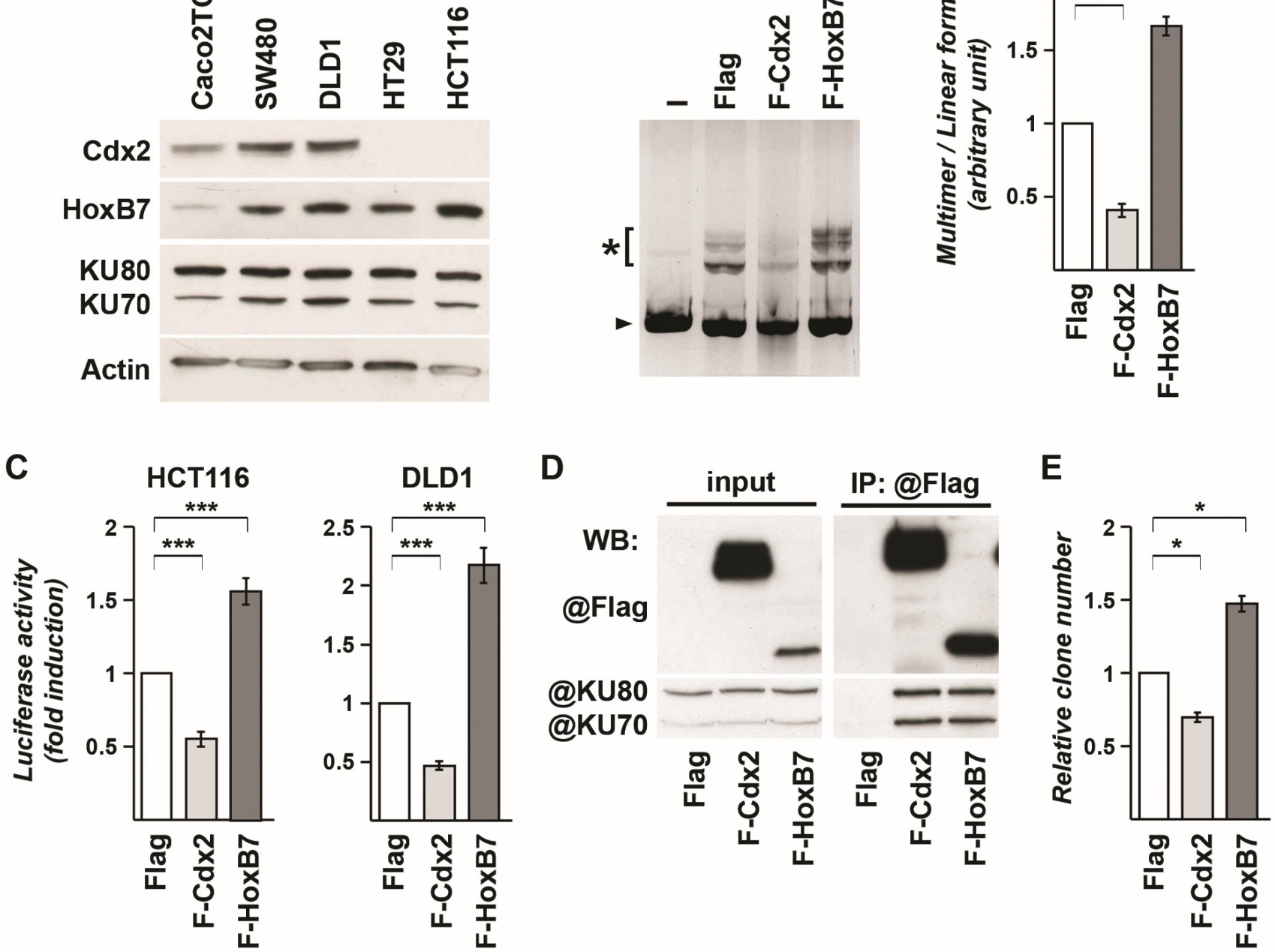


Figure 1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F-Cdx2
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Luciferase activity
(fold induction)
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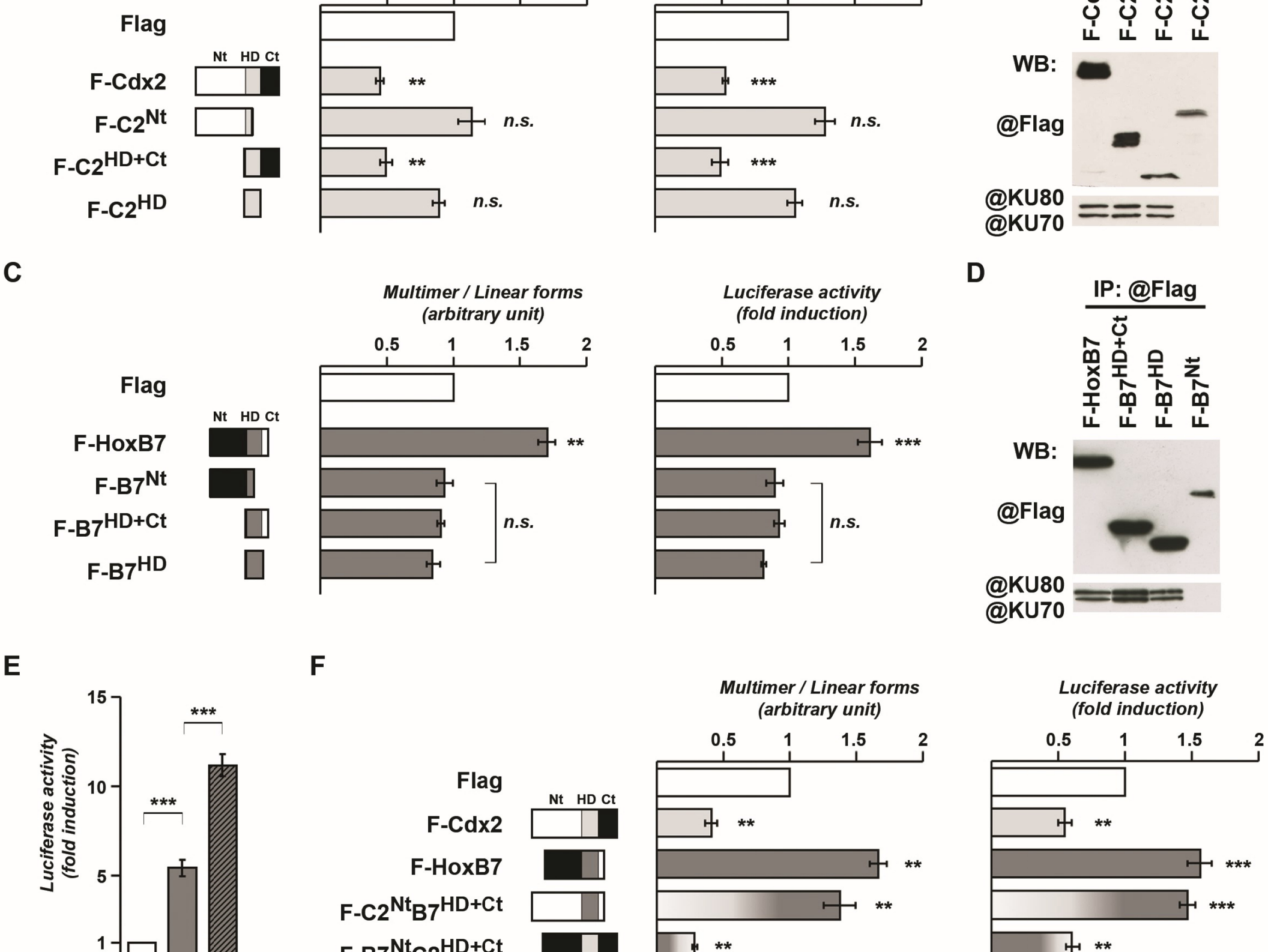
Figure 2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F-C2Nt
F-C2HD+Ct
F-C2HD
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Multimer / Linear forms
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Luciferase activity
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Luciferase activity
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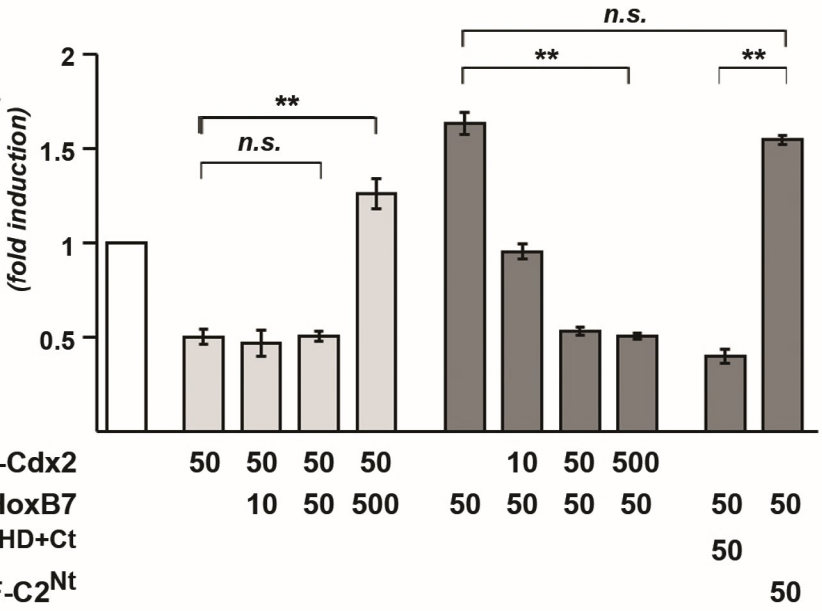
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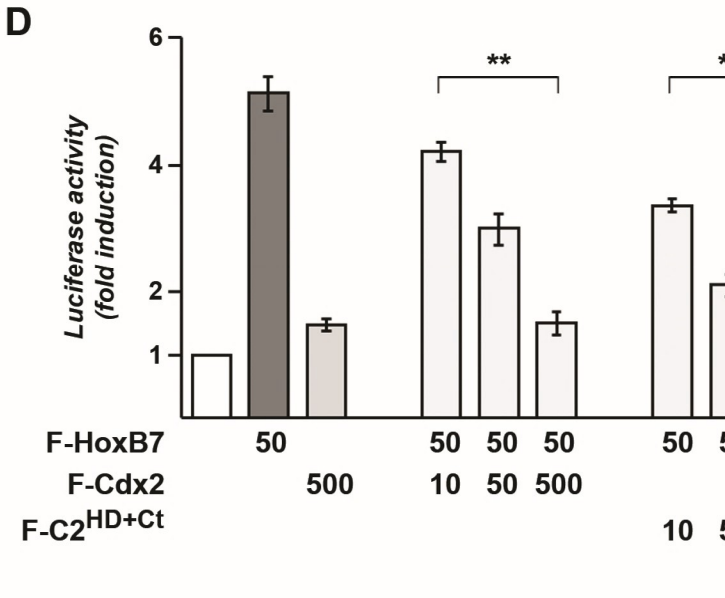
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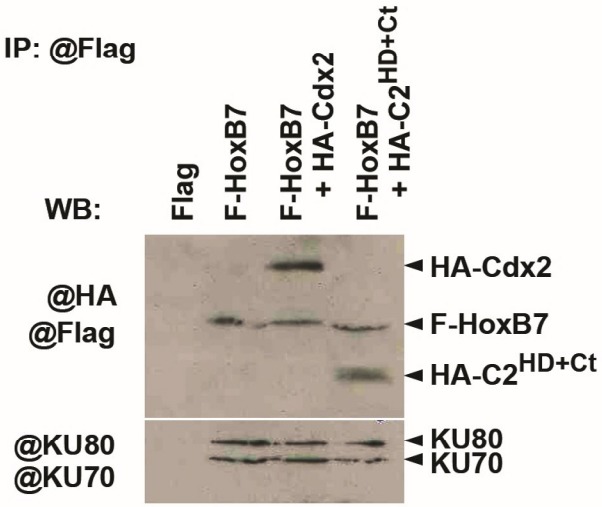
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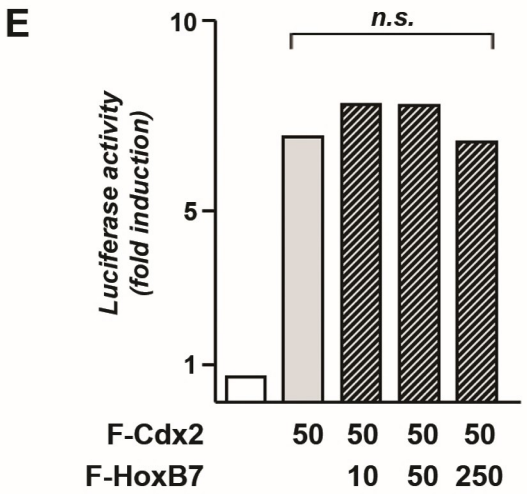
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Luciferase activity
(fold induction)
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F-HoxB7
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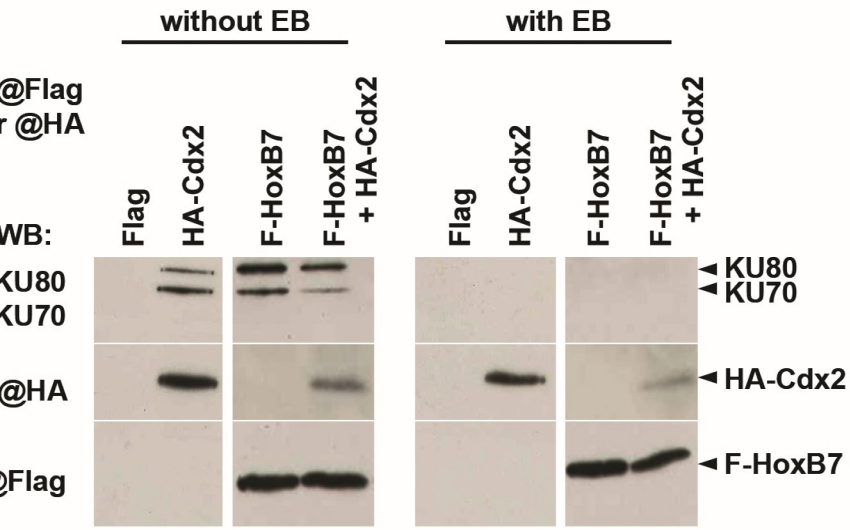
10 50 250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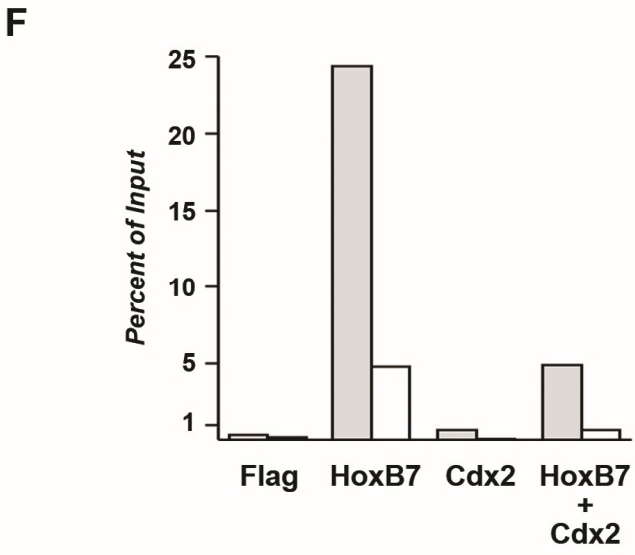
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Percent of Input
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HoxB7
+
Cdx2

Figure 4