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Interplay between KLF4 and ZEB2/SIP1 in the regulation of E-cadherin expression

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ABSTRACT

E-cadherin expression is repressed by ZEB2/SIP1 while it is induced by KLF4. Independent data from the literature indicate that these two transcription factors could bind close to each other in the proximal region of the E-cadherin gene promoter. We have here explored a potential competition between ZEB2 and KLF4 for the binding to the E-cadherin promoter. We show an inverse correlation between ZEB2 expression levels and KLF4 recruitment on the E-cadherin promoter in three breast cancer cell lines and in A431/HA.ZEB2 cells in which ZEB2 expression is induced by doxycycline (DOX). We identified a region of the E-cadherin promoter bound by KLF4 which is necessary for the activation of the E-cadherin promoter activity after KLF4 overexpression. This region is localized between positions −28 and −10 and thus overlaps with one of the ZEB2 binding sites. Deleting the bipartite ZEB2 binding site results in increased KLF4 induced E-cadherin promoter activity. Taken together, our results suggest that E-cadherin expression in cancer cells is controlled by a balance between ZEB2 and KLF4 expression levels.

1. Introduction

There is mounting evidence that epithelial-to-mesenchymal transition (EMT) contributes to the metastatic spread of epithelial tumor cells. At the molecular level, EMT is characterized by the down-regulation of "epithelial" markers (E-cadherin, Cytokeratins, and ZO-1) and the expression of "mesenchymal" markers (N-Cadherin, Vimentin, FSP1/S100A4) [1,2]. The phenotypical modifications characterizing EMT are mediated by specific transcription factors, among which the E-box binding transcription factor ZEB2 [3,4].

The downregulation of E-cadherin is today considered as a good indicator of EMT. E-cadherin is a major cell–cell adhesion molecule present in adherens junctions. E-cadherin abnormalities, leading to a reduction of intercellular cohesiveness, have been linked to tumor progression [5]. E-cadherin downregulation during tumor-associated EMT is mostly due to transcriptional inactivation through E-Boxes located in the minimal promoter [6]. This repression is mediated by specific transcription factors including ZEB2 [3,4].

ZEB2 is a transcription factor containing two zinc-finger clusters at the N- and C- terminal domains (NZF and CZF), and a central homeodomain (HD). It also contains a SMAD-binding domain (SBD) and a CtBP interaction domain (CID). ZEB2 binds as a monomer to two CACCT sequences (E-box) through its zinc-finger clusters [7,8]. ZEB2 downregulates E-cadherin expression by binding two E-boxes of the minimal E-cadherin promoter, located at 75 and 25 bp upstream of the transcription start site [9]. Mutation of one of the E-boxes leads to a partial derepression, while mutation of both E-boxes leads to a complete derepression of E-cadherin promoter activity. ZEB2 can repress E-cadherin expression independently of CtBP binding [10]. It has also been suggested that ZEB2 can prevent the binding of positive regulatory factors to the region located between the two E-Boxes [7,8]. However, the precise mechanism of E-cadherin repression by ZEB2 is still debated.

KLF4, a member of the Krüppel-like family of zinc-finger transcription factors has been reported to activate E-cadherin transcription [11]. KLF4 is expressed in a wide variety of human tissues in which it regulates different cellular functions such as proliferation, differentiation, apoptosis or homeostasis [12]. During cancer progression KLF4 could act either as a tumor promoter or as a suppressor, depending on the cancer type and the cellular context [13]. Thus, in invasive ductal breast carcinoma increased KLF4 nuclear staining is associated with poor prognosis [14]. However, KLF4 was recently shown to inhibit tumor progression in a xenograft animal model [15] and EMT *in vitro* [11]. KLF4 accordingly up-regulates E-cadherin expression. Promoter studies have

Abbreviations: EMT, epithelial-to-mesenchymal transition; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; DOX, doxycycline.

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indeed pointed out the importance of a −359 to +30 region of the E-cadherin promoter in this regulation [11] but the precise KLF4 binding site on the E-cadherin promoter has not been identified. KLF4 has been shown to bind CACCC sequences and GC-rich sequences in the promoter of i.e. KRT4, p21WAF1/CIP1 and CYP1A1 genes [16–19]. Interestingly, the E-cadherin promoter contains several CACCC sequences and a GC-rich region, located between the two ZEB2 binding sites.

Given the proximity between the ZEB2 binding sites and the putative KLF4 binding sites in the E-cadherin promoter and their opposite functions on E-cadherin expression, we have explored the possibility that KLF4 and ZEB2 could compete for the binding of the E-cadherin promoter.

2. Materials and methods

2.1. Plasmids

pcDNA3.1-HisB-GKLF was a gift from Dr. Rustgi [19]. ZEB2 cDNA was cloned by PCR from pEF6MycHis/ZEB2 [9] and inserted in the pTRE2pur vector (Clontech, Mountain View, CA, USA) in which HA epitope had been inserted. The promoter constructs containing fragments −93/−10, −93/−28, and −66/−10 of the E-cadherin promoter were generated by ligation of double stranded synthetic oligos in pGL3Prom vector (Promega, Madison, WI, USA). pGL2-Basic-EcadK1 (Addgene, Cambridge, MA, USA) was used to clone fragment −108/+19 of the E-cadherin promoter into pGL3Basic vector.

2.2. Cell lines

T47D, BT-20, MCF7, MDA-MB-231 and Hs578T were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and cultured as recommended. A431 expressing the Tet-On Advanced regulator (Clontech, Mountain View, CA, USA) were used to generate an A431 cell line expressing ZEB2 in the presence of doxycycline by stable transfection of the linearised pTRE2pur/HA.-ZEB2 vector with Fugene 6.

2.3. Antibodies

Rabbit anti-KLF4 (H180) used in ChIP and normal rabbit IgG used for EMSA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit IgG for ChIP control was purchased from Diagenode. Rabbit anti-KLF4 (AB4138) used in western blot was purchased from Millipore (Billerca, MA, USA). Mouse anti-β-actin (Ab8226) and rabbit anti-vimentin (Ab8545) were purchased from Abcam (Cambridge, UK). Mouse anti-HA (MMS-101R) was purchased from Covance (Princeton, NJ, USA). Mouse anti-E-cadherin (clone 36) was purchased from BD Transduction Laboratories (San Jose, CA, USA).

2.4. Chromatin immunoprecipitation

ChIP was performed as reported previously [20]. Chromatin was sheared for 15 min (30 s on/30 s off) with Biorupter (Diagenode, Liège, Belgium). For each immunoprecipitation reaction, 50 μg of pre-cleared chromatin and 5 μg of Rabbit anti-KLF4 (H-180) or control Rabbit IgG were used. Antibody/Antigen complexes were pulled-down by rec-Protein G-Sepharose® 4B Conjugate (Invitrogen, Camarillo, CA, USA). Recovered DNA was quantified by real-time PCR using Fast Start SYBR Green Master (Roche Applied Sciences). The gene specific primer sequences were : E-cadherin : 5′-GGCCGGCAGGTGAAC-3′ and 5′-GGGCTGGAGTCTGAACTGAC-3′ [21]; Prm1 : 5′-CACCTGGCCATGGTTTGTG-3′ and 5′-TGGAACTCTGTGGGCTGTG-3′. Experiment was repeated three times, and PCR were done in triplicate.

2.5. Reporter assay

30 000 cells were seeded in 24-wells plates. 200 ng of reporter vector and 200 ng of expression vector were co-transfected with Fugene 6 (Promega). 48 h after transfection, cells were lysed and harvested with 100 μL Passive Lysis Buffer (Promega). Activity was measured by mixing 50 μL of lysate with 100 μL of assay buffer (20 mM $MgCl_2$, 50 mM Glycylglycine pH 7.8, 10 mM ATP, 470 μM luciferine) in 96-wells flat bottom white plates. Luminescence was measured with Victor X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) and normalized to protein level. Experiment was repeated three times, with each condition in triplicate.

2.6. Electrophoretic mobility shift assay

KLF4 was produced *in vitro* using pcDNA3.1-HisB-GKLF with TnT T7 Quick Coupled Transcription/Translation System (Promega). Oligos were labeled with α-^{32}P by Klenow filling. EMSAs were carried by mixing 5 μL of Transcription/Translation product with 2 pmol of oligo in 20 μL binding reaction containing 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 5 μM $ZnCl_2$, 10% glycerol, 0,05% NP-40, 10 μg BSA and 1 μg of poly(dI-dC) (Sigma–Aldrich, St. Louis, MO, USA). After incubation at room temperature for 15 min, the samples were loaded on a 5% polyacrylamide gel and migrated at 140 V for 4 h. The gels were dried and exposed to CL-XPosure film (Thermo Scientific, Rockford, IL, USA) overnight. For competition experiments, cold oligos were incubated 15 min at room temperature prior to the addition of labeled oligos. For supershift experiments, 4 μg of antibody were incubated in the mixture for 2 h on ice prior to the addition of labeled DNA probe. The sequences in the sense orientation are shown below:

−66/−10: GCCAATCAGCGGTACGGGGGGCGGTGCCTCCGGGGCT CACCTGGCTGCAGCCACG

p21 [22] : GGCCCCGGGGAGGGCGGTCCCGGGCGGCGCGGTGGG

p21m : GGCCCCGGGGAGAATGGTCCCGAATGGCGCGGTGGG

2.7. Western blot analysis

Protein extraction and western blot analysis were performed according to a previously described protocol [23].

2.8. RNA Isolation and RT-PCR

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Sciences). RNA quantification was carried out on a Nano Drop 1000 (Thermo Fisher Scientific). Reverse-transcription was performed on 1 μg of total RNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences). PCRs were performed with the Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA).

Primers used were: ZEB2 : 5′-CACAGCTCTTCCACCTCAAAGC-3′ and 5′-TTTTGCGAGACAGACAGGAG-3′ ; GAPDH : 5′-GTGAAGGTCG-GAGTCAACG-3′ and 5′-GGTGAAGACGCCAGTGGACTC-3′ ; Vimentin 5′-GACAATGCGTCTCTGGCACGTCTT-3′ and 5′- TCCTCCGCCTCCTG CAGGTTCTT-3′ ; E-cadherin : 5′-CCCATCAGCTGCCCAGAAAATGAA-3′ and 5′- CTGTCACCTTCAGCCATCCTGTTT-3′ ; 28S rRNA 5′-GTTC ACCCACTAATAGGGAACGTGA-3′ and reverse 5′-GGATTCTGACTTAG AGGCGTTCAGT-3′.

2.9. Statistical analyses

Unpaired *t*-tests (two-tailed, two-sample unequal variance) were performed using OpenOffice Calc.

3. Results

3.1. KLF4 binds the E-cadherin promoter in the absence of ZEB2

We first examined the expression levels of ZEB2, KLF4 and E-cadherin in two EMT positive (Vimentin +, E-cadherin −) and three EMT negative (Vimentin −, E-cadherin +) breast cancer cell lines. As shown in Fig. 1A, KLF4 mRNA was detected in all examined breast cancer cell lines, irrespective of E-cadherin and vimentin expression. In contrast, as previously reported [9,24], ZEB2 was expressed in cells where E-cadherin expression is low or absent. To confirm the relation between ZEB2 expression and EMT we generated A431/HA.ZEB2 cells expressing HA-tagged ZEB2 under the control of a DOX-activated promoter. A431 cells overexpressing ZEB2 had indeed been used previously to study ZEB2-induced EMT [21,25]. As shown in Fig. 1C, A431/HA.ZEB2 cells displayed an "epithelial" phenotype and expressed KLF4 and E-cadherin but very low levels of ZEB2 (Fig. 1B). ZEB2 induction by DOX treatment resulted in the acquisition of a "mesenchymal" phenotype, the induction of vimentin and the downregulation of E-cadherin expression. Importantly, KLF4 protein levels were not affected (Fig. 1B). Thus, both in breast cancer cell lines and in the conditional A431 EMT model, E-cadherin levels are negatively correlated to ZEB2 levels.

In order to analyse the potential interplay between KLF4 and ZEB2 on the regulation of E-cadherin expression, we investigated, by Chromatin Immunoprecipitation (ChIP), KLF4 binding to the E-cadherin promoter in cells expressing high or low levels of ZEB2. First, we examined KLF4 binding to the E-cadherin promoter in the A431/HA.ZEB2 inducible model. KLF4 binding to the E-cadherin proximal promoter was detected in cells expressing low levels of ZEB2. However, DOX induction of ZEB2 expression inhibited KLF4 binding to the promoter (Fig. 2A). We used the *Prm1* promoter as a negative control for the specificity of KLF4 binding. *Prm1* codes for sperm protamin1, a protein only expressed during spermatogenesis and silenced in other contexts.

In order to strengthen our results obtained with the A431/HA.ZEB2 inducible model, we compared endogenous KLF4 binding to the E-cadherin promoter in three breast cancer cell lines expressing different levels of ZEB2 (Fig. 1A) and KLF4 (Fig. 2C). We could detect KLF4 binding to the E-cadherin promoter only in MCF7 cells (Fig. 2B), which do not express detectable levels of ZEB2. On the opposite, KLF4 was not immunoprecipitated with the E-cadherin proximal promoter in MDA-MB-231 and Hs578T cells which express ZEB2. These results suggest that ZEB2 and KLF4 bindings to the proximal E-cadherin promoter might be mutually exclusive.

3.2. KLF4 and ZEB2 have opposite effects on the E-cadherin promoter activity

We next investigated the regulation of the E-cadherin promoter activity in response to modulations of KLF4 and ZEB2 expression levels. We generated a luciferase reporter vector containing a fragment of the E-cadherin promoter extending from residues −108 to +19. This fragment contains the two E-boxes previously shown to constitute the bipartite ZEB2 binding site [9]. This vector was co-transfected with or without a KLF4 expression vector into MDA-MB-231 and A431/HA.ZEB2 cells. KLF4 overexpression induced a significant increase of luciferase activity in A431/HA.ZEB2 cells (Fig. 3A). However, after doxycycline treatment, KLF4 was unable to activate the promoter. In MDA-MB-231 cells, expressing ZEB2, KLF4 activated the E-cadherin promoter. This apparent discrepancy could be explained by the relatively moderate ZEB2 levels in MDA-MB231 compared to DOX-treated A431/HA.ZEB2 cells (Fig. 3B) which might not be sufficient to compete with KLF4 overexpression. These results indicate that the balance between ZEB2 and KLF4 protein levels is important for the modulation of E-cadherin expression.

3.3. KLF4 binds the E-cadherin promoter in vitro

Our ChIP results have shown that KLF4 binds the E-cadherin promoter. In order to localise more precisely a KLF4 binding site on the E-cadherin proximal promoter, different oligonucleotides spanning the −108 to +19 region of the E-cadherin promoter were screened as probes in EMSA (Electrophoretic Mobility Shift Assay) experiments. Since KLF4 binding sites are also recognized by Sp1 and other Krüppel-like family members, we chose to perform the EMSA with *in vitro*-produced KLF4 protein. KLF4 was able to shift only the oligonucleotide containing the −66/−10 fragment as shown by the representative result in Fig. 4A. The cold probe and a cold oligonucleotide containing a KLF4 binding site from the p21 gene promoter [22] competed with the −66/−10 fragment. The oligonucleotide containing a mutated KLF4 binding site from

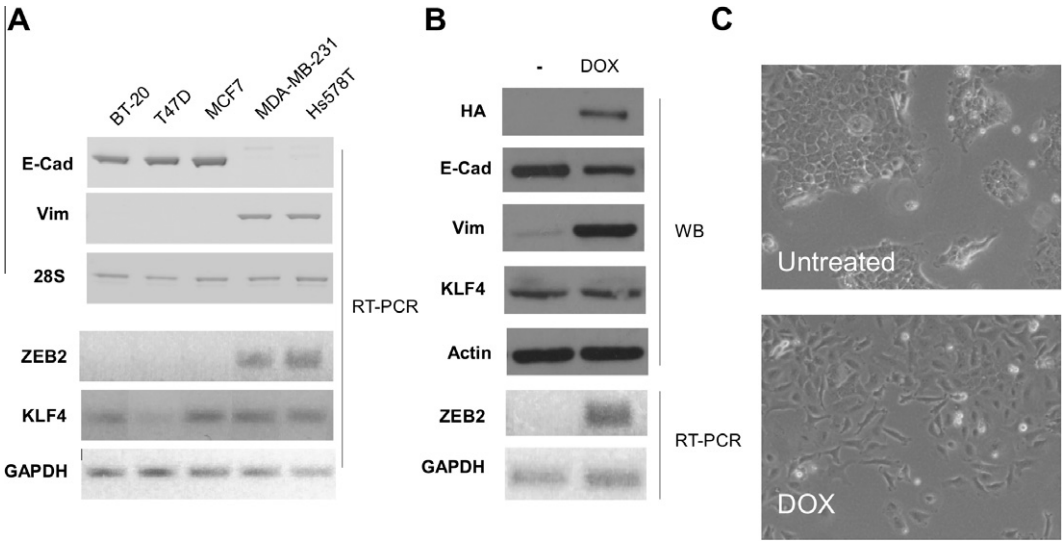

Fig. 1. (A) RT-PCR of E-cadherin, Vimentin, 28S rRNA, ZEB2, KLF4 and GAPDH on five breast cancer cell lines. (B) Western blot analysis of HA-tagged ZEB2, E-cadherin, Vimentin, KLF4 and actin in A431/HA.ZEB2 cells before and after DOX treatment. ZEB2 and GAPDH mRNA levels were detected by semi-quantitative RT-PCR performed on RNA extracted from the same cells. (C) Phase-contrast photography of A431/HA.ZEB2 cells untreated or treated with DOX for 48 h.

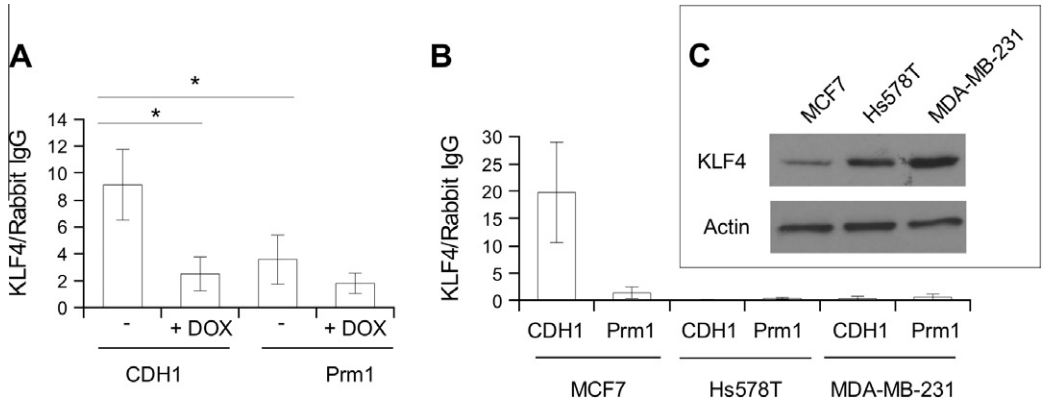

Fig. 2. (A) ChIP analysis of KLF4 binding to the E-cadherin promoter. Chromatin extracted from A431/HA.ZEB2 cells treated or not with DOX was immunoprecipitated with anti-KLF4 antibody or rabbit control serum (IgR). After DNA purification, quantitative PCR was performed to determine the KLF4 specific enrichment of the E-cadherin proximal promoter (CDH1) region. Prm1 promoter region was amplified as control for specific binding. (B) Endogenous KLF4 binding to E-cadherin promoter in the three three breast cancer cell lines used in A. (C) Western blot analysis of KLF4 protein level in three breast cancer cell lines used in the chromatin immunoprecipitation assay. * denotes $p < 0.01$.

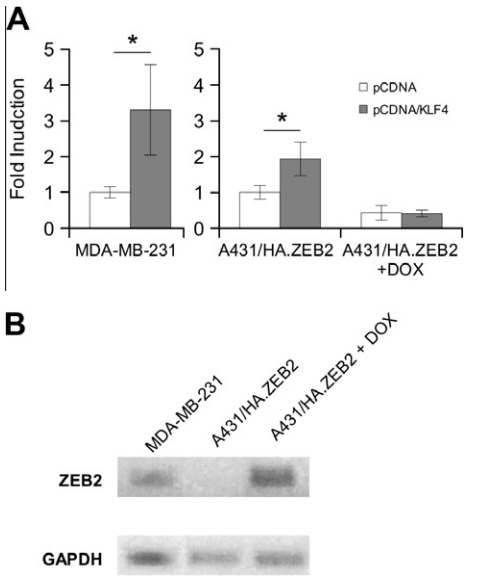

Fig. 3. (A) Luciferase activity measured in MDA-MB-231 and A431/HA.ZEB2 cells treated or not with DOX and cotransfected with pGL3Basic/E-Cad-108/+19 reporter vector and with pcDNA or pcDNA/KLF4 expression vector. Luciferase activity was normalized to total protein content. (B) RT-PCR analysis of ZEB2 expression in MDA-MB-231 and A431/HA.ZEB2 treated or not with DOX. * denotes $p < 0.01$.

the p21 promoter did not compete. A KLF4 specific antibody completely abolished the binding, indicating that the protein-DNA interaction was specific.

Interestingly, the −66/−10 fragment contains one of the ZEB2 binding sites. Remacle et al. have suggested that ZEB2 might exert its repressive function by preventing the access of activating factors to the sequence comprised between the two E-boxes [7]. Our data agree with this hypothesis and suggest that KLF4 might be one of the activating factors.

3.4. KLF4 activates the E-cadherin promoter if binding of ZEB2 to DNA is impaired

To further investigate this hypothesis, we cloned three shorter E-cadherin promoter regions in the pGL3-promoter vector, containing a strong SV40 promoter. The −93/−10 reporter vector contains the two E-boxes; the −93/−28 reporter contains only E-box 1, while only E-box 3 is present in the −66/−10 reporter vector (Fig. 4B). The reporters were co-transfected with a KLF4 expression vector in A431/HA.ZEB2 cells treated or not with doxycycline, and in MDA-MB-231 cells. Regarding ZEB2, we observed that ZEB2 expression inhibited the activity of the −93/−10 vector (Fig. 4C). Indeed, DOX-induced ZEB2 expression in A431/HA.ZEB2 cells did not modify the activity of the −93/−28 and −66/−10 vectors, containing one of the E-boxes in A431/HA.ZEB2. In accordance with the literature [21], these results confirm the importance of both E-boxes in mediating ZEB2 effects. Looking at KLF4 effects, we first observed that the −93/−28 promoter construct was not activated by KLF4. Together with the EMSA results (Fig. 4A), this suggests that the region from −28 to −10 mediates KLF4-induced activation. In A431/HA.ZEB2 cells, KLF4 cDNA transfection stimulated the activity of the −93/−10 and −66/−10 promoters in non-treated cells. Forced ZEB2 expression inhibited only the KLF4-dependent activation of the −93/−10 promoter fragment. This result indicates that KLF4-induced activation can be impaired only if both E-boxes are present. In MDA-MB-231 cells (expressing ZEB2), KLF4 overexpression similarly induced the activity of the −93/−10. The −66/−10 reporter was nevertheless also induced, again pointing out the importance of the balance between ZEB2 and KLF4 expression, as already suggested by the results of Fig. 3.

4. Discussion

In the present study, we have gathered indications that the regulation of E-cadherin expression during EMT process integrates opposite regulatory actions of ZEB2 and KLF4. Our results indeed show that KLF4 binds the E-cadherin promoter in a region overlapping with a known ZEB2 binding site and that these two factors have opposite effects on E-cadherin promoter activity.

Our results first showed that ZEB2 is expressed in cell lines negative for E-cadherin expression. This is in agreement with previous results associating high ZEB2 expression with high migratory/invasive properties of EMT-derived cells [21,24]. In contrast, we found KLF4 expression in all the cell lines we have analyzed, irrespective of E-cadherin expression levels. Literature data regarding KLF4 expression is quite controversial. For instance, diminished expression of KLF4 has been reported in several cancer types including esophageal cancer, prostate cancer and lung cancer while an overexpression has been described in primary breast ductal carcinoma and oral squamous carcinoma [14,26]. Also *in vitro*, overexpression of KLF4 has been associated with cancer stem cell attributes of

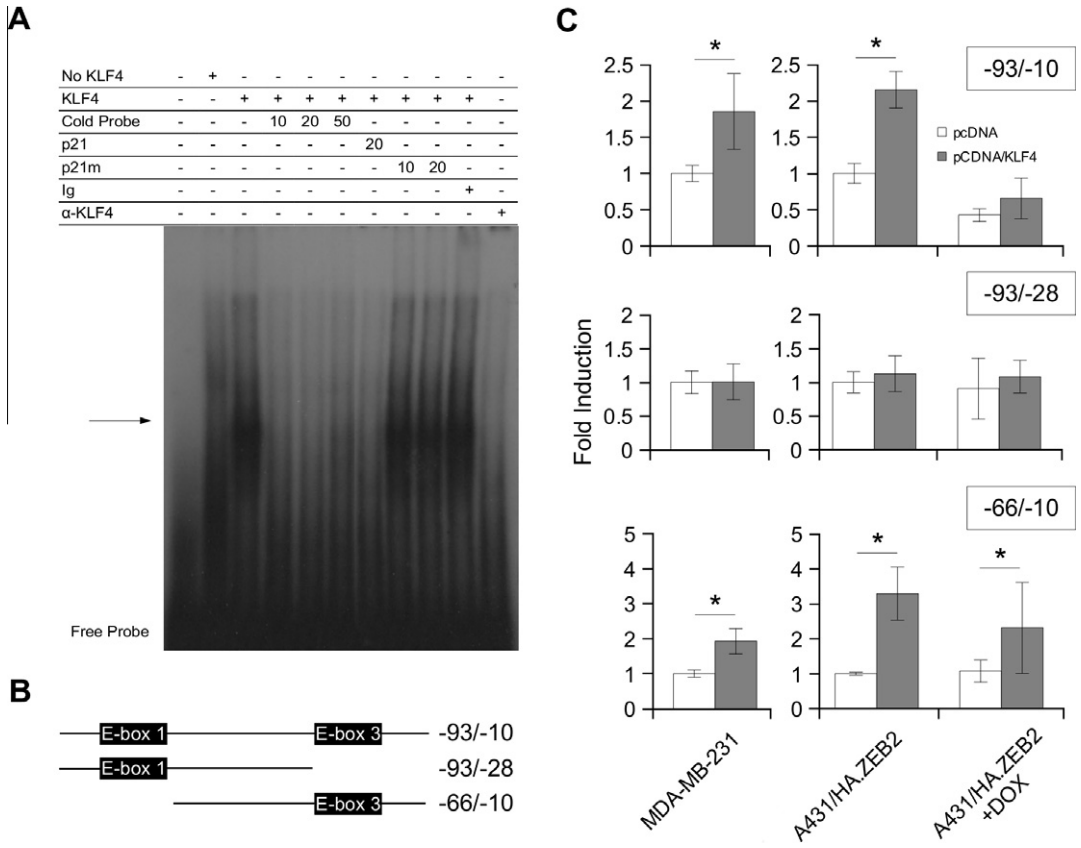

Fig. 4. (A) EMSA analysis performed with a probe spanning the region −66/−10 of the E-cadherin promoter. First lane : probe only. KLF4 : *in vitro*-produced KLF4. No KLF4 : *in vitro* production with empty vector. Cold probe : unlabeled −66/−10 (10, 20 and 50 indicate fold molar excess of competitor). p21 : specific competitor containing two GC-Boxes. p21m : p21 with mutated GC-Boxes (10 and 20 indicate fold molar excess of competitor). Ig : supershift control. α-KLF4 supershift with anti-KLF4 antibody. (B) Schematic representation of the three regions cloned for the luciferase assay depicted in C. (C) Luciferase activity measured in A431/HA.ZEB2 and MDA-MB-231 cells transfected with pGL3prom/E-Cad −93/−10, pGL3prom/E-Cad −93/−28 or pGL3prom/E-Cad_−66/−10. Luciferase activity was measured 48 h after transfection and normalized to total protein content. ∗ denotes $p < 0.01$.

breast tumor cells [27], attributes that have often been linked to EMT features. In contrast, KLF4 overexpression has been shown to induce E-cadherin expression and inhibit EMT in breast tumor cells [11]. Though such controversies are not clearly understood, it has to be pointed out that the balance between the expression levels of KLF4 and other factors (such as ZEB2) could influence its activity. This balance is likely a better key determinant of KLF4 transcriptional activity rather than its individual expression level.

Accordingly, we showed by ChIP an inverse correlation between ZEB2 expression and KLF4 binding to the E-cadherin promoter. Also, ZEB2 overexpression inhibited KLF4-induced activation of a proximal E-cadherin promoter (−108 to +19) construct in A431/HA.ZEB2 cells. When overexpressed, KLF4 was nevertheless able to transactivate this promoter region in MDA-MB-231 cells despite ZEB2 expression. This might indicate that the relatively low endogenous level of ZEB2 in this cell line cannot compete with the strongly overexpressed KLF4 in good agreement with the results of Yori et al. [11] showing that MDA-MB-231 stably transfected with a KLF4 expression vector reexpressed E-cadherin. Our results thus clearly support the importance of the interplay between ZEB2 and KLF4 for the regulation of E-cadherin expression and suggest that the balance between ZEB2 and KLF4 expression is important in E-cadherin expression regulation.

The interplay between ZEB2 and KLF4 in the regulation of E-cadherin expression is probably explained by the close vicinity of the binding sites for both factors. Indeed, our results confirm that two E-boxes are necessary for ZEB2-induced repression of E-cadherin expression, as reported previously [9,21]. On the other hand, Yori et al. previously showed that a −359 to +30 E-cadherin promoter fragment was activated by KLF4. Our results here refine the KLF4 binding site on the E-cadherin promoter. The EMSA results indeed located a KLF4 binding site between positions −66 and −10 of the E-cadherin promoter. Furthermore, reporter vector assays revealed that region extending from nucleotide −28 to −10 is necessary for E-cadherin promoter activation by KLF4. Interestingly, this −28 to −10 region overlaps with E-box 3, one of the bipartite binding sites of ZEB2. Supporting a functional consequence of this overlap, we further showed that KLF4 activation of the E-cadherin promoter is inhibited in the presence of ZEB2 only if the two E-boxes are present.

In conclusion, our results originally revealed an interplay between KLF4 and ZEB2 in E-cadherin regulation. They strongly support the previously proposed hypothesis that ZEB2 represses transcription by preventing the binding of an activating factor on the E-cadherin promoter [7] and identify KLF4 as such an activating factor.

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