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Homeodomain protein DLX4 counteracts key transcriptional control mechanisms of the TGF-β cytostatic program and blocks the anti-proliferative effect of TGF-β

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Abstract

The anti-proliferative activity of transforming growth factor-β (TGF-β) is essential for maintaining normal tissue homeostasis and is lost in many types of tumors. Gene responses that are central to the TGF-β cytostatic program include activation of the cyclin-dependent kinase inhibitors $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ and repression of c-myc. These gene responses are tightly regulated by a repertoire of transcription factors that include Smad proteins and Sp1. The *DLX4* homeobox patterning gene encodes a transcription factor that is absent from most normal adult tissues, but is expressed in a wide variety of malignancies including lung, breast, prostate and ovarian cancers. In this study, we demonstrate that DLX4 blocks the anti-proliferative effect of TGF-β. DLX4 inhibited TGF̃-β-mediated induction of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ expression. DLX4 bound and prevented Smad4 from forming complexes with Smad2 and Smad3, but not with Sp1. However, DLX4 also bound and inhibited DNA-binding activity of Sp1. In addition, DLX4 induced expression of c-myc independently of TGF-β/Smad signaling. The ability of DLX4 to counteract key transcriptional control mechanisms of the TGF-β cytostatic program could explain in part the resistance of tumors to the anti-proliferative effect of TGF-β.

Keywords

cell growth; homeobox genes; Smad; Sp1; TGF-β

INTRODUCTION

Transforming growth factor-β (TGF-β) mediates diverse processes such as proliferation, differentiation and apoptosis during embryogenesis and in adult tissues (Siegel and

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Massagué, 2003; Massagué, 2008). Upon binding of TGF-β, the TGF-β type II receptor (TGF-βRII) phosphorylates and activates the type I receptor (TGF-βRI). In turn, TGF-βRI phosphorylates Smad2 and Smad3 which translocate to the nucleus and form complexes with Smad4 and other DNA-binding factors to activate or repress transcription (Shi and Massagué, 2003; Feng and Derynck, 2005). In most types of normal cells, TGF-β inhibits cell proliferation by inducing arrest in G_1 phase. The gene responses that are central to the TGF-β cytostatic program are activation of the cyclin-dependent kinase inhibitors $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, and repression of the growth-promoting c-myc and ID transcription factors (Reynisdóttir *et al.*, 1995; Chen *et al.*, 2002; Kang *et al.*, 2003). The cytostatic program is tightly integrated by feedback loops that protect against competing mitogenic signals (Siegel and Massagué, 2003). For example, TGF-β activates $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ transcription via cooperative interactions between Sp1 and Smad proteins and also prevents repression of these genes by c-myc [Feng *et al.*, 2000; Pardali *et al.*, 2000; Gartel *et al.*, 2001; Feng *et al.*, 2002].

Resistance to TGF-β̃-mediated growth-inhibition is an important feature in the pathogenesis of a wide variety of tumors (Siegel and Massagué, 2003; Massagué, 2008). In several types of tumors, this resistance has been attributed to mutations or deletion of core components of the TGF̃-β signaling pathway. Inactivating mutations in TGF-βRII have been reported in 60–90% of colon cancers associated with microsatellite instability (MSI) (Markowitz *et al.*, 1995; Watanabe *et al.*, 2001). Mutations or deletions of Smad4 occur in ~50% of pancreatic and non-MSI colorectal cancers (Hahn *et al.*, 1996; Woodford-Richens *et al.*, 2001). By contrast, TGF-βRII mutations have not been detected in lung cancers with MSI (Takenoshita *et al.*, 1997), and the frequency of Smad4 mutations in lung cancers is low (7%) (Nagatake *et al.*, 1996). Similarly, many breast and prostate cancers are resistant to TGF-β–mediated growth-inhibition, but rarely contain TGF-βRII or Smad mutations (Schutte *et al.*, 1996; Takenoshita *et al.*, 1998; Bello-DeOcampo and Tindall, 2003). TGF-β receptor mutations occur in 12–31% of ovarian cancers (Wang *et al.*, 2000; Francis-Thickpenny *et al.*, 2001), but many TGF-β̃-resistant ovarian cancers have been found to express functional receptors (Yamada *et al.*, 1999). Resistance of many tumors to TGF-β-mediated growth-inhibition therefore cannot solely stem from mutations or deletions of core components of the TGF̃-β signaling pathway and likely arise from other aberrations.

Homeobox genes encode transcription factors that control cell differentiation (McGinnis and Krumlauf, 1992). *DLX4* (also reported as *BP1*) (Haga *et al.*, 2000) is a member of the *DLX* family of homeobox genes that controls many aspects of embryonic development including bone morphogenesis and skeletal patterning (Panganiban and Rubenstein, 2002). Although absent from most normal adult tissues, DLX4 is widely expressed in leukemias, lung, breast, ovarian and prostate cancers (Haga *et al.*, 2000; Tomida *et al.*, 2007; Man *et al.*, 2005; Hara *et al.*, 2007; Schwartz *et al.*, 2009). Because DLX4 is expressed in diverse types of tumors, we considered the possibility that DLX4 controls a pathogenic mechanism common to multiple organ sites. Our study demonstrates that DLX4 blocks gene responses that are central to the TGF-β cytostatic program by sequestering Smad4 and Sp1, and by inducing c-myc. This blocking activity of DLX4 could explain in part why tumor cells are resistant to the anti-proliferative effect of TGF-β.

RESULTS

DLX4 blocks TGF-β-induced, Smad-dependent responses

To determine whether DLX4 blocks the anti-proliferative effect of TGF-β, we initially used the non-tumorigenic lung epithelial cell line Mv1Lu. Mv1Lu is a well-established model for studying TGF-β-mediated induction of $p15^{Ink4B}$ expression and growth arrest (Reynisdóttir *et al.*, 1995; Feng *et al*, 2002). Whereas growth of Mv1Lu cells was inhibited by TGF-β in a dose-dependent manner, enforced expression of DLX4 in these cells increased resistance to TGF-β [Figure 1A]. Expression of DLX4 in Mv1Lu cells blocked the ability of TGF-β to induce $p15^{Ink4B}$ expression [Figure 1B]. Cell cycle analysis revealed that DLX4 expression inhibited the induction of G_1 arrest in Mv1Lu cells by TGF-β [Figure 1C]. Enforced expression of DLX4 in the non-tumorigenic mammary epithelial cell line NMuMG also inhibited TGF-β-induced G_1 arrest [Supplementary Figure 1]. In converse experiments, we determined whether knockdown of DLX4 in tumor cells increased TGF-β-mediated growth-inhibition using shRNAs that targeted different sites of *DLX4* (sh90, sh92). The ability of shRNAs to knockdown DLX4 in MCF-7 breast cancer cells was confirmed by Western blot [Figure 1D] and also by immunofluorescence staining and qPCR [Supplementary Figures 2A,B]. Knockdown of DLX4 in MCF-7 cells was observed to increase sensitivity to TGF-β in cell viability assays [Figure 1E], and also increased the proportion of cells in G_1 phase [Figure 1F].

Because TGF-β can also inhibit growth by Smad-independent mechanisms (Petritsch *et al.*, 2000), we used the Smad4-deficient MDA-MB-468 breast cancer cell line to confirm that DLX4 blocks Smad-dependent growth-inhibition. Growth of MDA-MB-468 cells was not inhibited by TGF-β [Figure 1G]. On the other hand, reconstitution of Smad4 in these cells increased responsiveness to TGF-β [Figure 1G]. This responsiveness to TGF-β was abrogated by DLX4 [Figure 1G]. Expression of $p21^{WAF1/Cip1}$ was induced by TGF-β in MDA-MB-468 cells reconstituted with Smad4, and this induction was blocked by DLX4 [Figure 1H]. Enforced expression of DLX4 induced c-myc levels, irrespective of the absence or presence of Smad4 [Figure 1H]. We further investigated the effect of DLX4 on c-myc induction by assaying c-*myc* promoter activity. Activity of the c-*myc* promoter was repressed by TGF-β in control Mv1Lu cells [Figure 2A]. In contrast, expression of DLX4 in Mv1Lu cells induced c-*myc* promoter activity irrespective of TGF-β signaling [Figure 2A]. Conversely, knockdown of DLX4 in MCF-7 cells inhibited c-*myc* promoter activity and increased sensitivity to TGF-β–mediated repression [Figure 2B]. These results suggest that DLX4 blocks TGF-β/Smad-dependent repression of c-myc expression, and also induces c-myc levels independently of TGF-β/Smad signaling.

The first 113 bp of the $p15^{Ink4B}$ promoter are essential for induction by TGF-β and contain two Smad-binding elements (SBEs) (Feng *et al.*, 2000). Activity of this minimal promoter region was induced by TGF-β in control Mv1Lu cells, and this induction was abolished by mutation of the SBEs [Figure 2C]. Expression of DLX4 in Mv1Lu cells abolished the induction of wild-type $p15^{Ink4B}$ promoter activity by TGF-β [Figure 2C]. DLX4 also modestly inhibited activity of the SBE-mutant promoter [Figure 2C]. To confirm that DLX4 blocks Smad-dependent transcription, we performed reporter assays using MDA-MB-468

cells. Whereas wild-type p15^{Ink4B} promoter activity was unresponsive to TGF-β in Smad4-deficient MDA-MB-468 cells, responsiveness was conferred when Smad4 was expressed in these cells. This Smad4-dependent responsiveness was eliminated when DLX4 was co-expressed [Figure 2D].

In addition to its anti-proliferative effect, TGF-β is well-known to induce epithelial-to-mesenchymal transition (EMT) (Siegel and Massagué, 2003; Massagué, 2008). Because DLX4 abrogated TGF-β-mediated growth inhibition, DLX4 might also block the ability of TGF-β to induce EMT. To address this question, we used the NMuMG cell line, a well-established model for studying TGF-β-induced EMT. Smad4 has been demonstrated to be essential for TGF-β-induced EMT in several cell types including NMuMG cells (Deckers *et al.*, 2006). TGF-β treatment of vector-control NMuMG cells induced profound epithelial-to-fibroblastic morphologic transformation [Supplementary Figure 3A], loss of E-cadherin and up-regulation of N-cadherin [Supplementary Figures 3B,3C]. Enforced expression of DLX4 blocked down-regulation of E-cadherin and induction of N-cadherin [Supplementary Figures 3B,3C]. Epithelial morphology was considerably retained in TGF-β-treated +DLX4 NMuMG cells [Supplementary Figure 3A]. Together, our findings that DLX4 blocks TGF-β-mediated, Smad-dependent growth-inhibition and EMT indicate that DLX4 inhibits a core component of the TGF-β/Smad signaling pathway.

DLX4 blocks transcriptional activity of TGF-β-activated R-Smads

Smad-dependent transcription is controlled at multiple levels, including phosphorylation and nuclear localization of receptor-regulated Smads (R-Smads) (Shi and Massagué, 2003). DLX4 did not alter levels of Smad2 expression or Smad2 phosphorylation [Figure 3A]. DLX4 also did not interfere with nuclear translocation of Smad2 following TGF-β stimulation [Figure 3B]. DLX4 was predominantly localized in the nucleus and its localization was not affected by TGF-β stimulation [Supplementary Figures 4A,B]. These findings indicate that DLX4 likely inhibits nuclear events downstream of the TGF̃-β signaling pathway.

We determined whether DLX4 inhibits transcriptional activity of TGF-β receptor-regulated Smads. A construct was generated in which the GAL4 DNA-binding domain (DBD) was fused to the linker region and MH2 transcriptional activation domain of Smad2 [amino acids 173 to 467]. This chimera was co-expressed in Mv1Lu cells with a firefly luciferase (F-Luc) reporter controlled by five tandem GAL4 binding sites. Transcriptional activity of GAL4-Smad2 was induced by TGF-β, and this activation was abolished when DLX4 was expressed [Figure 3C]. Similar results were obtained using the hepatoma cell line HepG2 [Figure 3C]. Expression of DLX4 also abolished TGF-β-mediated activation of a chimera comprising the GAL4-DBD fused to the linker region and MH2 domain of Smad3 [amino acids 133 to 425] [Figure 3D]. Conversely, knockdown of DLX4 in MCF-7 cells increased TGF-β-mediated induction of GAL4-Smad2 and GAL4-Smad3 activity [Figures 3E,F].

Smad2 and Smad3 serve as substrates for the TGF-β receptors, whereas other R-Smads (Smads 1, 5 and 8) are utilized by the bone morphogenetic protein (BMP) and anti-Müllerian receptors (Shi and Massagué, 2003; Feng and Derynck, 2005). We initially tested the ability of DLX4 to inhibit transcription induced by other members of the TGF super-

family by using a synthetic promoter comprising four tandem SBEs (pSBE4-Luc). DLX4 blocked induction of this promoter by TGF-β and by BMP-4 [Figure 3G]. TGF-β- and BMP-specific R-Smads preferentially bind distinct DNA sequences (Kusanagi *et al.*, 2000; Korchynskyi and ten Dijke, 2002). Indeed, BMP-4 was not as effective as TGF-β in inducing pSBE4-Luc activity [Figure 3G]. We further tested the effect of DLX4 on BMP-induced transcription by using a reporter plasmid that contained the BMP-responsive promoter of the *Id1* gene. DLX4 blocked BMP-induced *Id1* promoter activity [Supplementary Figure 5]. The blocking effect of DLX4 was confirmed by using a synthetic promoter comprising two tandem copies of *Id1* BMP response elements (BRE-Luc) [Figure 3H]. Because TGF-β- and BMP- specific R-Smads utilize Smad4 as the common and essential partner for formation of functional transcriptional complexes (Shi and Massagué, 2003), our findings raise the possibility that DLX4 inhibits Smad4.

DLX4 prevents Smad4 from binding R-Smads

We subsequently determined whether DLX4 interferes with the binding of Smad4 to R-Smads. Following transfection with FLAG-tagged DLX4 or empty vector, HepG2 cells were treated with or without TGF-β. Smad2 was immunoprecipitated, and precipitates analyzed by immunoblotting using Ab to Smad4. Binding of Smad4 to Smad2 was inhibited when DLX4 was expressed [Figure 4A]. We confirmed these findings by immunoprecipitating Smad4 and detecting Smad2 in precipitates [Figure 4A]. DLX4 also prevented binding of Smad4 to Smad3 [Figure 4A]. DLX4 did not alter Smad expression levels [Figure 4B]. These results indicate that DLX4 likely inhibits transcriptional activity of Smad2 and Smad3 by preventing Smad4 from interacting with these R-Smads.

Binding affinity and selectivity of Smad complexes for target gene promoters is governed by interactions with other DNA-binding factors (Shi and Massagué, 2003; Feng and Derynck, 2005). The minimal $p15^{Ink4B}$ promoter contains two Sp1 binding sites adjacent to the SBEs, and its induction by TGF-β involves binding of Smad4 to Sp1 as well as to R-Smads (Feng *et al.*, 2000). As reported by others (Feng *et al.*, 2000; Pardali *et al.*, 2000), TGF-β stimulation induced binding of Smad4 to Sp1 in –DLX4 cells. This binding was not inhibited by DLX4 [Figure 4C]. Surprisingly, we observed increased association of Smad4 with Sp1 in +DLX4 cells in the absence of TGF-β stimulation [Figure 4C]. DLX4 did not alter expression of Sp1 [Figure 4B]. To determine whether DLX4 alters formation of Smad-Sp1-DNA complexes, we performed DNA pull-down assays using a biotinylated oligonucleotide that contained nt −108 to −39 of the $p15^{Ink4B}$ promoter and included the two Sp1 binding sites and SBEs [Figure 4D]. Increased levels of Smads were detected in DNA-protein complexes when –DLX4 cells were stimulated with TGF-β [Figure 4E]. In contrast, this induction was inhibited in +DLX4 cells [Figure 4E]. DLX4 also inhibited interaction of Sp1 with the $p15^{Ink4B}$ promoter [Figure 4E]. To confirm the blocking activity of DLX4 in a more physiological context, we performed chromatin immunoprecipitation (ChIP) assays. As shown in Figure 4F, association of R-Smads, Smad4 and Sp1 with the $p15^{Ink4B}$ promoter was detected by ChIP assays in −DLX4 cells following TGF-β treatment. In contrast, interactions of Smad and Sp1 proteins with the $p15^{Ink4B}$ promoter were abrogated in +DLX4 cells [Figure 4F]. Together, these findings raise the possibility that DLX4 binds Smads and/or Sp1.

DLX4 directly binds Smad4

To initially investigate whether DLX4 associates with Smad4 in cells, IP assays were performed using extracts of HepG2 cells that expressed FLAG-DLX4. DLX4 associated with Smad4 in the absence and presence of TGF-β stimulation [Figure 5A]. Association of endogenous DLX4 with Smad4 was detected in IP assays using extracts of MCF-7 cells [Figure 5B]. This interaction was abrogated when *DLX4* shRNA was expressed in these cells [Figure 5B].

To determine whether DLX4 interacts with Smad4 by direct binding, we tested the ability of *in vitro*-translated ^{35}S-labeled DLX4 to bind GST-Smad4 protein. GST-pulldown assays demonstrated that DLX4 directly binds Smad4 [Figure 5C]. DLX4 also bound Smad2, albeit more weakly [Figure 5C]. We sought to identify the Smad4-binding domain of DLX4 by testing truncated GST-DLX4 fusion proteins for their ability to bind *in vitro*-translated ^{35}S-labeled Smad4 [Figure 5D]. Deletion of the C-terminal tail of DLX4 only weakly affected its ability to bind Smad4 [Figure 5E]. In contrast, deletion of the DNA-binding homeodomain of DLX4 markedly inhibited its Smad4-binding ability. Binding of the DLX4 homeodomain to Smad4 was detected but not as strongly as observed with full-length DLX4 [Figure 5E]. We also investigated which domain of Smad4 interacts with DLX4 by testing the ability of GST-DLX4 protein to bind *in vitro*-translated portions of Smad4 protein [Figure 5D]. Direct binding of DLX4 was detected to the Smad4 MH1 domain, but not to the MH2 domain [Figure 5F].

DLX4 also directly binds Sp1

Because DLX4 directly binds Smad4 which also binds Sp1, we determined whether DLX4 interacts with Sp1. In IP assays using lysates of transfected HepG2 cells, DLX4 was found to associate with Sp1 [Figure 6A]. This association was not dependent on TGF-β stimulation. We confirmed the ability of endogenous DLX4 to interact with Sp1 in IP assays using extracts of MCF-7 cells [Figure 6B]. GST-DLX4 protein directly bound to *in vitro*-translated ^{35}S-labeled Sp1 [Figure 6C]. Whereas the N-terminal domain of DLX4 did not bind Sp1, binding by the homeodomain was strongly detected [Figure 6C]. We also tested the ability of GST-DLX4 protein to directly bind *in vitro*-translated ^{35}S-labeled portions of Sp1 [Figure 6D]. DLX4 did not bind the N-terminal domain of Sp1 [amino acids 1 to 557], but bound to its C-terminal DNA-binding domain [amino acids 557 to 778] [Figure 6E]. In the presence of DLX4, decreased levels of Sp1 were detected in complexes associated with the p15^{Ink4B} promoter in both oligonucleotide pull-down and ChIP assays [Figures 4E,4F]. In gel-shift assays, we observed that DLX4 did not bind p15^{Ink4B} promoter sequences, but prevented Sp1 from binding the promoter [Figure 6F]. These observations are consistent with the ability of DLX4 to partially inhibit activity of the SBE-mutant p15^{Ink4B} promoter that contains intact Sp1 binding sites [Figure 2C]. Enforced expression of DLX4 also inhibited activity of a synthetic promoter that comprised tandem Sp1-binding sites [Figure 6G]. Conversely, knockdown of DLX4 stimulated Sp1-driven promoter activity [Figure 6G]. These results indicate that DLX4 inhibits p15^{Ink4B} transcription by sequestering Sp1, in addition to preventing Smad4 from interacting with R-Smads.

DISCUSSION

The TGF-β cytostatic program is essential for maintaining normal tissue homeostasis and is tightly regulated by a network of transcription factors that include Smad proteins, Sp1 and c-myc (Siegel and Massagué, 2003; Massagué, 2008). In this study, we report that DLX4, a homeodomain protein that is expressed in a broad range of malignancies, blocks the anti-proliferative effect of TGF-β by counteracting key transcriptional control mechanisms of the TGF-β cytostatic program.

Our studies indicate there are several mechanisms by which DLX4 inactivates transcriptional control of the TGF-β cytostatic program. One mechanism is by sequestering Smad4 and preventing Smad4 from binding R-Smads. Smad interactions might also be prevented by binding of DLX4 to R-Smads as we observed binding, albeit weak, of DLX4 to Smad2. Because Smad4 and R-Smads interact with one another via their MH2 domains (Shi and Massagué, 2003), our finding that DLX4 binds the Smad4 MH1 domain is surprising. One explanation could be that binding of DLX4 to the MH1 domain induces a conformational change such that the MH2 domain of Smad4 is unable to interact with R-Smads.

Transcription factors encoded by homeobox genes are characterized by their helix-turn-helix DNA-binding homeodomain (McGinnis and Krumlauf, 1992). Our findings that binding of DLX4 to Smad4 is mediated in part through its homeodomain raises the question of specificity. Indeed, Hoxc8 interacts with Smad1 through its homeodomain (Yang *et al.*, 2000). DLX1 has been reported to bind Smad4, but its binding region and mechanism have not been defined (Chiba *et al.*, 2003). Although the homeodomain is conserved throughout the homeobox gene family, the specificity of family members for different Smads and different Smad domains is striking. DLX3 binds Smad6 but not Smad4 (Berghorn *et al.*, 2006). Hoxc8 interacts with the MH1 domain of Smad1 (Yang *et al.*, 2000), whereas Hoxa13 binds the MH2 domains of Smad1, Smad2 and Smad5 but does not bind Smad4 (Williams *et al.*, 2005). Within the homeodomain, the residues of the third helix are the most highly conserved. Less conserved residues of the first and second helices might govern preferential binding to a specific Smad protein or Smad domain. Although other homeodomain proteins might potentially block TGF-β-mediated growth inhibition by binding Smads, the specificity of this inhibition could largely depend on the context on their expression. Whereas most homeobox genes are expressed in a highly tissue-specific manner, DLX4 is expressed across diverse malignancies (Haga *et al.*, 2000; Man *et al.*, 2005; Hara *et al.*, 2007; Tomida *et al.*, 2007; Schwartz *et al.*, 2009). No other homeobox gene has been reported to be commonly expressed in tumors of lung, breast, ovarian, prostate and hematologic origin. Interference by DLX4 of TGF-β-mediated growth inhibition could therefore be a mechanism common to multiple organ sites.

Binding affinity and selectivity of Smad complexes for target gene promoters is principally dictated by interactions with other DNA-binding factors (Shi and Massagué, 2003, Feng and Derynck, 2005). Similarly, binding affinity and selectivity of several homeodomain proteins has been reported to be modulated by interactions with other transcriptional regulators (Shen *et al.*, 1999; Boogerd *et al.*, 2008). Our study is the first report that demonstrates that a

homeodomain protein directly interacts with Sp1 and modulates Sp1 activity. Our findings indicate that DLX4 directly binds the DNA-binding domain of Sp1 and impairs the DNA-binding ability of Sp1, but does not prevent Sp1 from associating with Smad4. Because DLX4 binds to Smad4 and to Sp1, we speculate that DLX4 inhibits $p15^{Ink4B}$ transcription in part by forming an inactive DLX4-Smad-Sp1 complex. Because transcription of $p21^{WAF1/Cip1}$ is also induced by TGF-β via cooperative interactions between Sp1 and Smad proteins (Pardali *et al.*, 2000), DLX4 could likely inhibit $p21^{WAF1/Cip1}$ transcription by a similar mechanism.

Our finding that DLX4 induces expression of c-myc independently of TGF-β signaling has two implications. Firstly, induction of c-myc provides a competing mitogenic signal against the TGF-β cytostatic program. Secondly, DLX4-induced c-myc expression might lead to down-regulated expression of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, since transcription of these genes is repressed by c-myc (Feng *et al.*, 2000; Gartel *et al.*, 2001). The repression of $p15^{Ink4B}$ transcription by c-myc has a notable similarity to our observations with DLX4. Feng *et al.* likewise reported that c-myc does not compete with Sp1 for interaction with Smads (Feng *et al.*, 2002). These authors speculated that c-myc forms an inactive complex with Sp1 and Smad proteins. However, in contrast to our findings with DLX4, c-myc does not inhibit interactions between Smad4 and R-Smads nor inhibits transcriptional activity of R-Smads *per se* (Feng *et al.*, 2002). Furthermore, unlike DLX4, c-myc does not affect binding of Sp1 to the $p15^{Ink4B}$ promoter (Feng *et al.*, 2002). DLX4 might therefore repress $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ transcription by increasing c-myc expression, and also by directly interfering with Smad4-R-Smad interactions and Sp1 DNA-binding activity.

Homeobox genes play essential roles in controlling cell differentiation during embryonic development. Increasing evidence indicates that aberrant expression of specific sets of homeobox genes in tumors can deregulate cell growth. For example, *HOXA5* regulates *p53* transcription and is silenced in breast cancers (Raman *et al.*, 2000). Overexpression of *HOXB7* and *HSIX1* in ovarian and breast tumors induces expression of fibroblast growth factor-2 and cyclin A1, respectively (Naora *et al.*, 2001; Coletta *et al.*, 2004). Aberrant expression of homeobox genes in tumors is thought to reflect an inappropriate recapitulation of embryonic pathways (Abate-Shen, 2002; Samuel and Naora, 2005). The ability of DLX4 to block TGF-β signaling might be related to the functions of *DLX* genes in controlling bone morphogenesis and skeletal patterning (Panganiban and Rubenstein, 2002). These processes are tightly regulated by members of the TGF super-family. Because DLX4 binds Smad4, DLX4 also likely blocks signaling emanating from other receptors of the TGF super-family. Indeed, DLX4 inhibited induction of transcriptional activity by BMP-4 [Figures 3G,3H; Supplementary Figure 5]. Several cross-regulatory interactions have been reported between BMPs and *DLX* genes during normal cell differentiation. BMP-2 activates *Dlx3* transcription (Park and Morasso, 2002), whereas Smad6, an antagonist of BMP signaling, inhibits DLX3 transcriptional activity (Berghorn *et al.*, 2006). Interestingly, we observed that levels of DLX4 protein decreased in cells following TGF-β stimulation [Figure 1H]. DLX4 might be a component of a regulatory loop that blocks TGF-β signaling and is conversely regulated by TGF-β. This feedback mechanism might play an important role in controlling normal embryogenesis and homeostasis.

Resistance to TGF-β̃-mediated growth-inhibition is an important feature in the pathogenesis of most types of tumors. This resistance has been attributed to TGF-β receptor or Smad mutations in several types of tumors, particularly those of gastrointestinal and pancreatic origin (Markowitz *et al.*, 1995; Hahn *et al.*, 1996; Watanabe *et al.*, 2001; Woodford-Richens *et al.*, 2001). Resistance of tumor cells to the anti-proliferative effect of TGF-β can also stem from down-regulation of TGF-β receptor expression (Tokunaga *et al.*, 1999) and $p15^{Ink4B}$ deletion (Gemma *et al.*, 1996). The ability of DLX4 to disable key transcriptional control mechanisms of the TGF-β cytostatic program could explain why tumors that lack aberrations in core components of the TGF̃-β signaling pathway can become resistant to the anti-proliferative effect of TGF-β.

A striking aspect of the TGF-β signaling pathway in tumors is its biphasic function. Many tumors are resistant t o the anti-proliferative effect of TGF-β but retain other TGF-β-mediated mechanisms that promote EMT and metastasis (Siegel and Massagué, 2003; Massagué, 2008). It has been thought that core components of the TGF-β pathway remain functional in these tumors, whereas downstream aberrations (such as $p15^{Ink4B}$ deletion) disable the growth-inhibitory arm of the pathway (Siegel and Massagué, 2003; Massagué, 2008). By sequestering Smad4, DLX4 inactivates the core pathway and might also block the metastasis-promoting function of TGF-β. Indeed, DLX4 markedly but not completely inhibited TGF-β-induced EMT in NMuMG cells [Supplementary Figures 3A–C]. The ability of DLX4 to inhibit TGF-β-induced EMT could explain the association of DLX4 with favorable prognosis in lung cancer patients and its metastasis-suppressive activity reported by Tomida *et al.* (2007). However, DLX4 levels in ovarian and breast cancers have been reported to correlate with disease progression (Hara *et al.*, 2007; Man *et al.*, 2005). There are several possible explanations for this paradox. TGF̃-β not only induces EMT by Smad-dependent mechanisms, but also via Smad-independent pathways that involve MAP kinase and RhoA activation (Moustakas and Heldin, 2005). TGF-β-induced, non-Smad pathways that promote cell migration might not be inhibited by DLX4. DLX4 could also promote tumor progression by other mechanisms such as sustained induction of c-myc. In addition, we have found that DLX4 promotes tumor angiogenesis by inducing expression of vascular endothelial growth factor and fibroblast growth factor-2 (Hara *et al.*, 2007). The mechanism that gives rise to overexpression of DLX4 in tumors is unclear. The *DLX4* gene maps to the 17q21.3-q22 region, a chromosomal ‘hot-spot’ that is amplified in ~10% of breast and ovarian cancers (Hyman *et al.*, 2002). However, DLX4 overexpression occurs in >50% of these tumors (Hara *et al.*, 2007; Man *et al.*, 2005) indicating that gene amplification is not the sole mechanism underlying this overexpression.

Like the Hedgehog, Wnt and Notch signaling pathways, the homeobox gene network is increasingly thought to be an important hub in the intimate relationship between embryonic development and cancer. Developmental patterning is known to be governed by interactions between homeobox genes and members of the TGF super-family, but this is the first report that functionally links a homeobox gene that is aberrantly expressed in tumors with resistance to the cytostatic activity of TGF-β. Future studies of pathways controlled by this intriguing class of patterning regulators will provide important insights into the multiple steps of the neoplastic process.

MATERIALS AND METHODS

Plasmids

The *DLX4* cDNA is described elsewhere (Haga *et al.*, 2000; Hara *et al.*, 2007). *DLX4* fragments were subcloned into pET41 GST vectors (Novagen) as described in the text. *DLX4* and non-targeting shRNAs were purchased from OriGene Technology. GST-Smad2 and GSTSmad4 plasmids were provided by Fang Liu (Rutgers University). *Smad* cDNAs were purchased from OriGene Technology and subcloned into the pFA-CMV plasmid containing the GAL4 DBD (Stratagene) as described in the text. The GAL4-driven pRF-Luc reporter construct was purchased from Stratagene. The pGL2 F-Luc reporter vector was purchased from Promega. The pBV-Luc, pSBE4-Luc and pBV-MYC(Del4) reporter constructs (Zawel *et al.*, 1998; He *et al.*, 1998) were provided by Bert Vogelstein (Johns Hopkins University) (Addgene plasmids 16539, 16495 and 16604). *Sp1* cDNA was provided by Keping Xie (M.D. Anderson Cancer Center). The Cignal Sp1 reporter construct was purchased from SABiosciences. F-Luc constructs containing wild-type and mutant $p15^{Ink4B}$ promoter sequences (Feng *et al.*, 2000) were provided by Xiao-Fan Wang (Duke University Medical Center) and Xin-Hua Feng (Baylor College of Medicine). The *Id1* promoter construct was provided by Robert Benezra (Memorial Sloan-Kettering Cancer Center) (Addgene plasmid 16048) (Tournay *et al.*, 1996). The BRE-Luc reporter construct (Korchynskyi and ten Dijke, 2002) was provided by Peter ten Dijke (Netherlands Cancer Institute).

Antibodies and other reagents

Sources of Abs were as follows: DLX4 (Abcam, Abnova); Smad2, phospho Smad2 (Ser465/467), Smad3, Smad4, $p15^{Ink4b}$, c-myc (Cell Signaling Technology); $p21^{WAF1/Cip1}$ (Calbiochem); Sp1, E-cadherin (Zymed); N-cadherin (BD Biosciences); actin, FLAG (Sigma-Aldrich); Smad2/3, lamin A/C (Santa Cruz Biotechnology). Recombinant Sp1 protein, TGF-β and BMP-4 were purchased from Promega, Sigma-Aldrich and R&D Systems, respectively.

Cell lines and transfection

Sources of cell lines were as follows: Mv1Lu, MDA-MB-468 and NMuMG (American Type Culture Collection); HepG2, Ampho-293 and MCF-7 (Michelle Barton, Douglas Boyd and Francois-Xavier Claret, M.D. Anderson Cancer Center). For transient expression, cells were transfected using FuGENE6 reagent (Roche). For generating stable lines, FLAG-tagged *DLX4* cDNA was subcloned into the pRetroQ vector (Clontech) and the retroviral construct was used to transfect Ampho-293 cells. Supernatants were harvested 2 d thereafter and used to infect target cells. Stable lines were selected by puromycin (0.5 μg/ml).

Growth assays and cell cycle analysis

Cells (4,000 per well) were seeded in 96-well plates, and cultured for 2 d in complete medium containing TGF-β at concentrations ranging from 0 to 100 ng/ml. Proliferation was measured by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche). For cell cycle analysis, cells were seeded in 10 cm dishes to reach 30% confluence

the following day. Cells were serum-starved overnight and then cultured in complete medium with and without addition of TGF-β for 18 h. Cells were harvested, fixed in 70% ethanol and distribution throughout the cell cycle determined by flow cytometric analysis of propidium iodide-staining.

Reporter assays

Cells were plated in 12-well plates and co-transfected with expression plasmid (400 ng), F-Luc reporter plasmid (100 ng), and pRL-CMV Renilla luciferase (R-Luc) reporter plasmid (0.5 ng)(Promega) for normalizing transfection efficiency. At 24 h after transfection, cells were cultured for an additional 18 h without and with TGF-β or BMP-4. Luciferase activities were assayed using the Dual-luciferase reporter assay kit (Promega).

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. Cells were stained for 1 h with Abs to Smad2, DLX4, E-cadherin or FLAG (1:200) and staining detected by Alexa Fluor 594-conjugated secondary Ab (Invitrogen).

IP and immunoblotting

Whole cell extracts were prepared by lysing cells in M-PER buffer (Pierce Biotechnology). Nuclear extracts were prepared by lysing cells in cell lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT plus protease and phosphatase inhibitors), followed by centrifugation at 800 × g for 10 min. Nuclear pellets were lysed in nuclear buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA), centrifuged at 12,000 × g for 10 min and supernatants collected. Nuclear extracts were pre-cleared with protein G agarose. 500 μg of nuclear extract was incubated with Ab for 4 to 12 h at 4°C. Immunoprecipitates were washed and subjected to SDS-PAGE and immunoblotting.

Oligonucleotide pull-down assay

Nuclear extracts were pre-cleared with streptavidin agarose for 30 min at 4°C and incubated overnight with biotinylated oligonucleotide corresponding to nt - 108 to −39 of the $p15^{Ink4B}$ promoter [Figure 4D]. Beads were washed with binding buffer (10 mM HEPES pH 7.9; 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5% NP-40). DNA-bound proteins were eluted and subjected to SDS-PAGE and immunoblotting.

ChIP

ChIP assays were performed using the ChIP Assay kit (Upstate Biotechnology) following the manufacturer's protocol with modifications. Cells were cross-linked using 1% formaldehyde and then neutralized with glycine. Cells were lysed in SDS lysis buffer and sonicated to generate DNA fragments of 200–1000 bp in length. Sheared chromatin was pre-cleared with protein G-agarose and incubated overnight with 4 μg of Sp1 and Smad Abs. Protein-DNA complexes were precipitated with protein G-agarose, followed by elution of immunoprecipitated complexes and reversal of cross-links. Purified DNA was used in PCR reactions to amplify a 535 bp fragment of the $p15^{Ink4B}$ promoter using the following

primers: sense, 5'-TATGGTTGACTAATTCAAACAG-3'; antisense, 5'-GCAAAGAATTCCGTTTTCAGCT-'3. The amplified fragment was confirmed by DNA sequencing.

In vitro binding assays

GST-DLX4 and GST-Smad fusion proteins were produced in *E. Coli*. ^{35}S-labeled DLX4, Smad4 and Sp1 were synthesized by *in vitro* transcription/translation (Promega) from the T7 promoter, and incubated for 2 h with 1 μg of GST fusion protein bound to glutathione-Sepharose beads (GE Healthcare). Beads were washed with binding buffer (20 mM Tris-HCl pH 8.0; 100 mM NaCl; 0.1% NP-40; 2 mM EDTA). Associated proteins were subjected to SDS-PAGE and visualized by autoradiography.

Gel-shift assay

Recombinant Sp1 (100 ng) was incubated with increasing amounts of *in vitro* translated FLAG-DLX4 and FLAG-tag at 23°C for 20 min in binding buffer (10 mM Tris-HCl pH 7.5; 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, 1 μg poly(dIdC). poly(dI-dC). 1 ng of ^{32}P-labeled oligonucleotide containing sequences −88 to −64 of the p15^{Ink4B} promoter [Figure 4D] was added to the binding reaction and incubated for an additional 20 min. DNA-protein complexes were separated by PAGE and visualized by autoradiography.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

Ab	antibody
BMP	bone morphogenetic protein
DBD	DNA-binding domain
EMT	epithelial-to-mesenchymal transition
F-Luc	firefly luciferase
IP	immunoprecipitation
R-Luc	Renilla luciferase
R-Smad	receptor-regulated Smad
TGF-β	transforming growth factor-β

TGF-βRI	TGF-β type I receptor
TGF-βRII	TGF-β type II receptor

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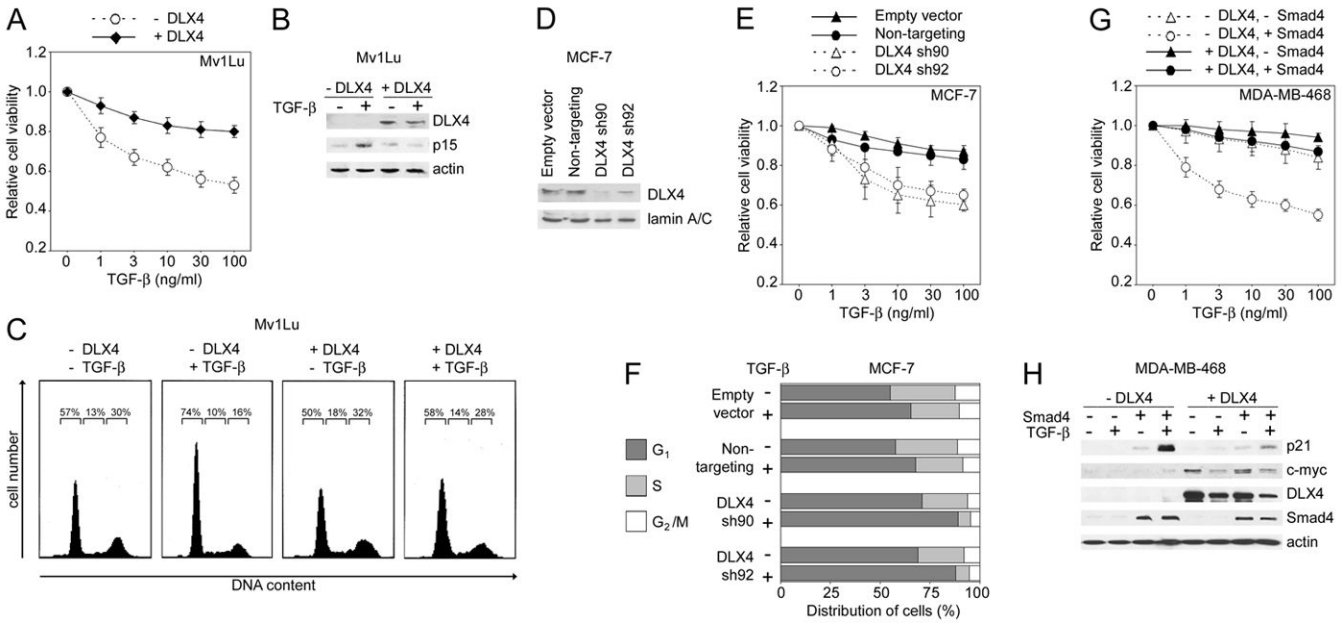

Figure 1.

DLX4 blocks TGF-β̃-mediated growth-inhibition. [A] Vector-control (−DLX4) and +DLX4 stable Mv1Lu lines were cultured with the indicated concentrations of TGF-β for 2 d. Changes in cell growth were determined by MTT assay, and expressed relative to growth of cells incubated without TGF-β. Shown are results of two independent experiments each performed in triplicate. [B] Western blot analysis of Mv1Lu lines following treatment without and with TGF-β (10 ng/ml) for 16 h. [C] Mv1Lu lines were treated without and with TGF-β for 18 h. Indicated are the proportions of cells in G_1, S and G_2/M phases determined by flow cytometric analysis of propidium iodide-staining. [D] MCF-7 cells were transfected with empty vector, non-targeting shRNA and *DLX4* shRNAs (sh90, sh92). At 2 d after transfection, DLX4 levels were assayed by Western blot. [E] Transfected MCF-7 cells were cultured with the indicated concentrations of TGF-β for 2 d. Changes in cell growth were determined by MTT assay. [F] Transfected MCF-7 cells were treated without and with TGF-β (10 ng/ml) for 18 h, and proportions of cells in cell cycle phases determined thereafter. [G] Vector-control (−DLX4) and +DLX4 stable MDA-MB-468 lines were transfected with Smad4. At 24 h thereafter, cells were cultured without and with TGF-β (10 ng/ml) for 2 d and changes in cell growth examined by MTT assay. [H] Western blot analysis of MDA-MB-468 lines following treatment without and with TGF-β for 16 h.

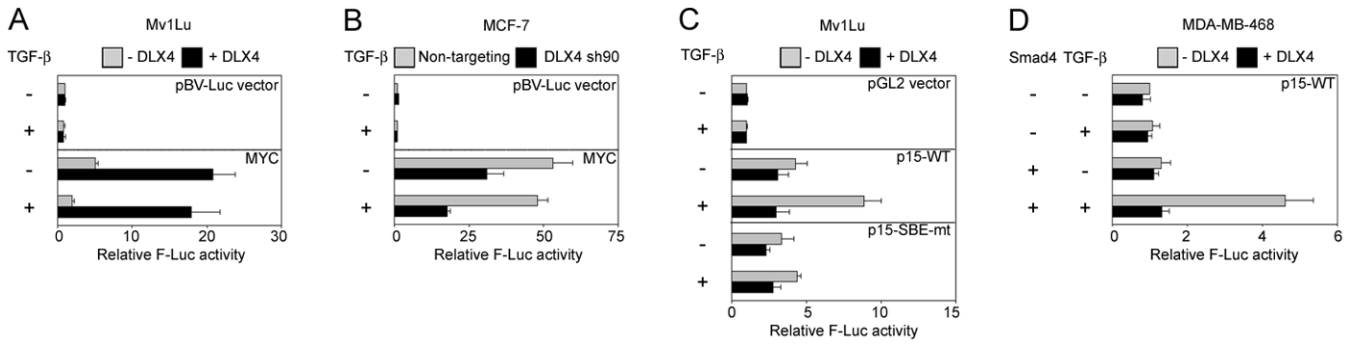

Figure .

DLX4 induces c-*myc* promoter activity and inhibits TGF-β̃-mediated induction of $p15^{Ink4B}$ promoter activity. [A] Mv1Lu cells were co-transfected with empty vector (grey bar) or DLX4 (black bar), together with empty pBV-Luc vector or with pBV-MYC(Del4) reporter plasmid that contains 900 bp of c-*myc* P1 and P2 promoter sequences. Transfected cells were cultured without and with TGF-β (10 ng/ml) for 18 h, and assayed for F-Luc activity. [B] Reporter assays for c-*myc* promoter activity were likewise conducted using MCF-7 cells that were co-transfected with non-targeting shRNA (grey bar) and *DLX4* (sh90) shRNA (black bar). [C] Mv1Lu cells were co-transfected with empty vector (grey bar) or DLX4 (black bar), together with reporter plasmids containing no promoter (pGL2 vector), $p15^{Ink4B}$ promoter sequences (−113 to +70)(p15-WT), and $p15^{Ink4B}$ promoter with mutated SBEs (p15-SBE-mt). Cells were cultured without and with TGF-β for 18 h, and assayed for F-Luc activity. [D] Reporter assays using the p15-WT reporter plasmid were performed using transfected MDA-MB-468 lines. Shown are relative F-Luc activities in three independent experiments each performed in duplicate. Values were normalized by activity of co-transfected R-Luc.

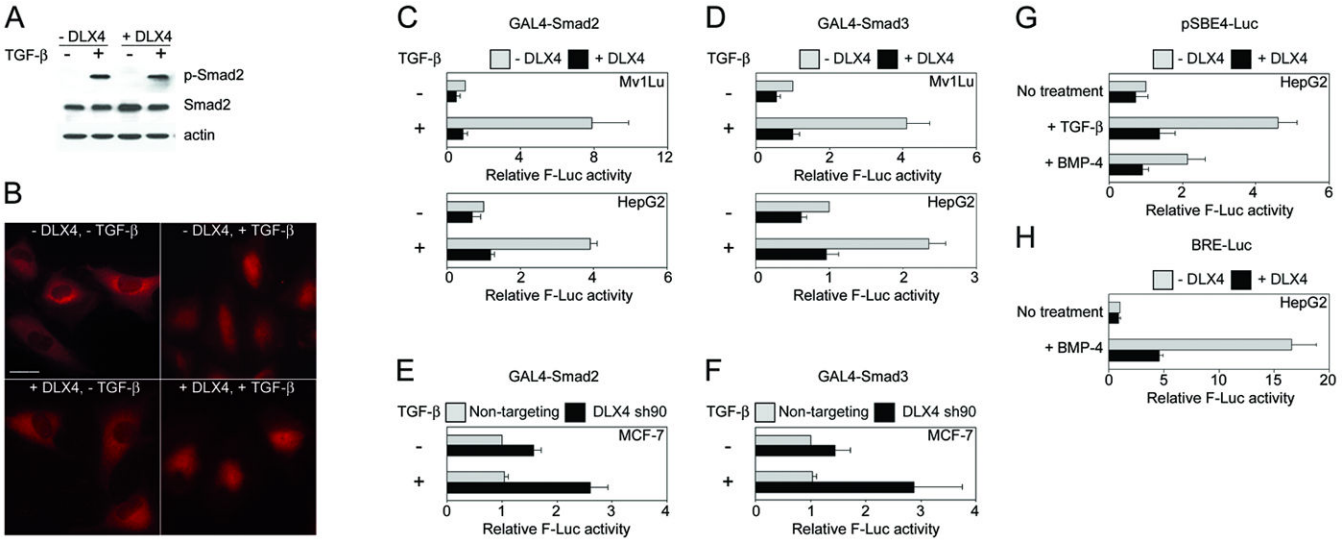

Figure 3.

DLX4 blocks Smad transcriptional activity. Vector-control (−DLX4) and +DLX4 Mv1Lu lines were serum-starved overnight and then treated without and with TGF-β (10 ng/ml) for 30 min. [A] Total and phosphorylated Smad2 were detected by Western blot. [B] Intracellular localization of Smad2 was detected by immunofluorescence staining. Bar, 20 μm. [C,D] Mv1Lu and HepG2 cells were co-transfected with empty vector (grey bar) or DLX4 (black bar), together with GAL4-driven F-Luc reporter plasmid and with [C] GAL4-Smad2 or [D] GAL4-Smad3. Transfected cells were cultured without and with TGF-β for 18 h, and assayed for F-Luc activity. [E] GAL4-Smad2 and [F] GAL4-Smad3 activities were likewise assayed in MCF-7 cells that were co-transfected with non-targeting shRNA (grey bar) or *DLX4* (sh90) shRNA (black bar). [G,H] HepG2 cells were co-transfected with empty vector (grey bar) or DLX4 (black bar) together with [G] SBE-driven pSBE4-Luc or [H] BRE-Luc reporter plasmids, and then cultured without and with TGF-β (10ng/ml) or BMP-4 (80 ng/ml) for 18 h. Shown are relative F-Luc activities in three independent experiments each performed in duplicate and normalized by activity of co-transfected R-Luc.

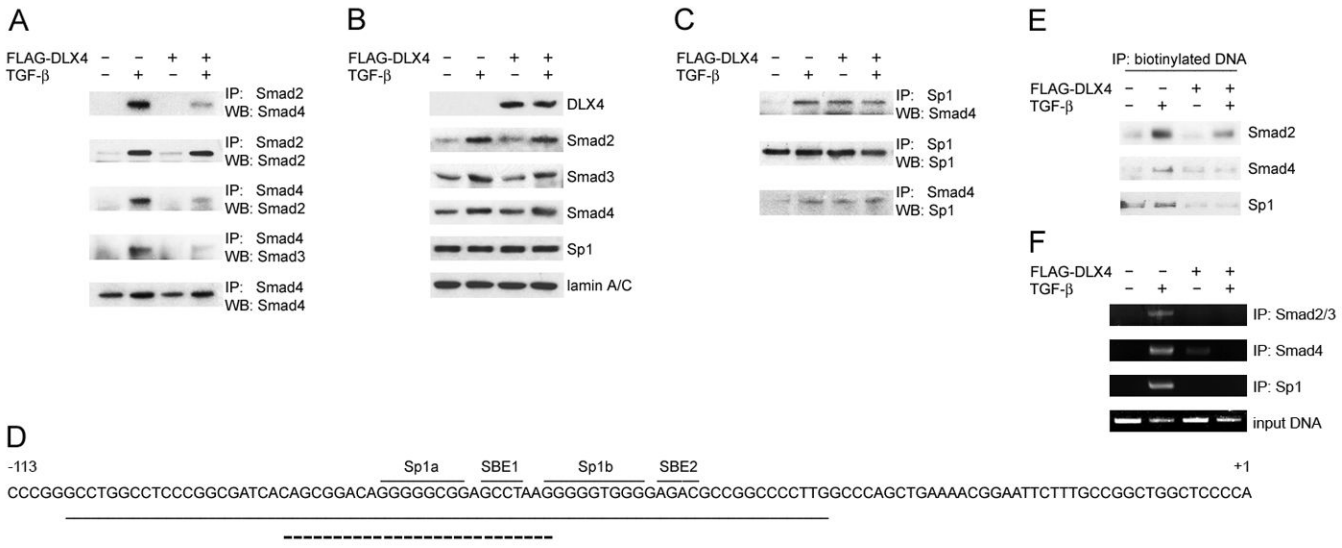

Figure 4.

DLX4 prevents Smad4 from binding R-Smads and blocks interactions of Smad and Sp1 proteins with the p15^{Ink4B} promoter. HepG2 cells were transfected with empty vector or with FLAG-tagged DLX4. At 24 h thereafter, cells were serum-starved overnight and then treated without and with TGF-β (10ng/ml) for 30 min. [A] Smad2 was immunoprecipitated from nuclear extracts and precipitates analyzed by immunoblotting using Ab to Smad4. Conversely, Smad4 was pulled-down and precipitates analyzed by immunoblotting using Smad2 Ab. Because HepG2 cells express low levels of Smad3, IP assays to detect binding of Smad3 to Smad4 were performed using extracts of cells that had been transfected with Smad3. [B] Western blot of DLX4, Smad proteins and Sp1 in nuclear extracts. [C] Sp1 was immunoprecipitated from nuclear extracts and precipitates analyzed by immunoblotting using Ab to Smad4. Conversely, Smad4 was pulled-down and Sp1 detected in precipitates. [D] Sequence of the minimal p15^{Ink4B} promoter indicating Sp1 binding sites and SBEs (adapted from Feng *et al.*, 2000). Underlined are sequences contained in the oligonucleotides used for oligonucleotide pull-down assays (solid line) and gel-shift assays (dashed line). [E] Biotinylated oligonucleotide containing sequences −108 to −39 of the p15^{Ink4B} promoter was incubated with HepG2 nuclear extracts and pulled-down. DNA-bound proteins in precipitates were analyzed by immunoblotting. [F] ChIP analysis of interactions of Smad and Sp1 proteins with the p15^{Ink4B} promoter. The input fraction corresponded to 1% of the chromatin solution of each sample before IP.

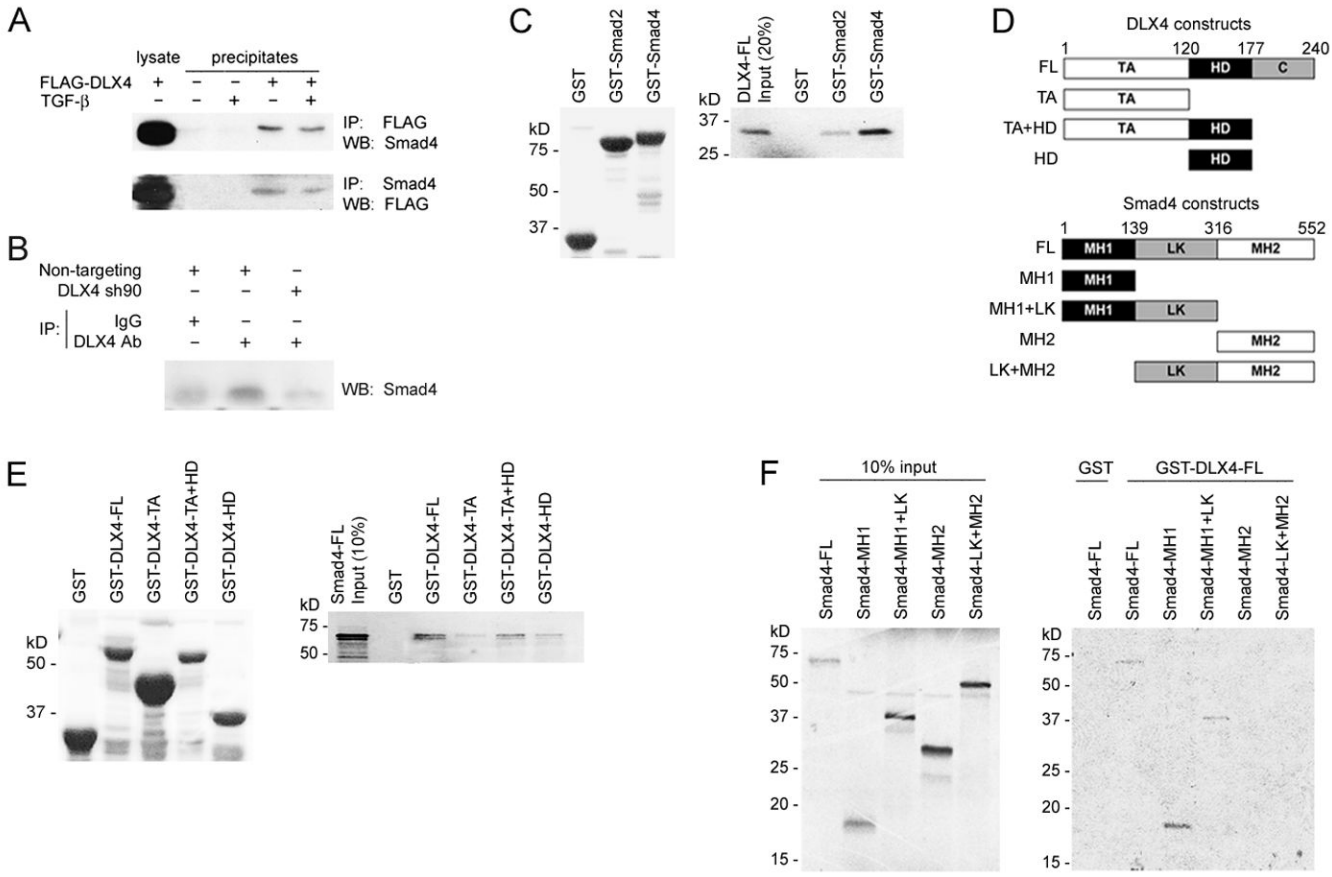

Figure 5.

DLX4 binds to Smad4. [A] HepG2 cells were transfected with empty vector or with FLAG-tagged DLX4. At 24 h thereafter, cells were serum-starved overnight and then treated without and with TGF-β (10ng/ml) for 30 min. FLAG-DLX4 was immunoprecipitated using FLAG Ab, and precipitates analyzed by immunoblotting using Ab to Smad4. Conversely, FLAG-DLX4 was detected in precipitates following IP using Smad4 Ab. [B] MCF-7 cells were transfected with non-targeting shRNA or with *DLX4* (sh90) shRNA. Endogenous DLX4 was immunoprecipitated using DLX4 Ab, and precipitates analyzed by immunoblotting using Smad4 Ab. IP using mouse IgG was included as a negative control. [C] Expression of GST-Smad2 and GST-Smad4 proteins was confirmed by SDS-PAGE *(left)*. GST-fusion proteins were assayed for direct binding to *in vitro* translated ^{35}S-labeled full-length DLX4 *(right)*. [D] GST-DLX4 constructs comprising the transactivation domain (TA), homeodomain (HD) and C-terminal tail (C), and Smad4 constructs comprising MH1 and MH2 domains and linker (LK) region. [E] Full-length (FL) DLX4 and portions thereof were expressed as GST-fusion proteins *(left)*, and assayed for binding to ^{35}S-labeled full-length Smad4 *(right)*. [F] ^{35}S-labeled full-length and truncated Smad4 were translated *in vitro (left)* and assayed for binding to full-length GST-DLX4 protein *(right)*.

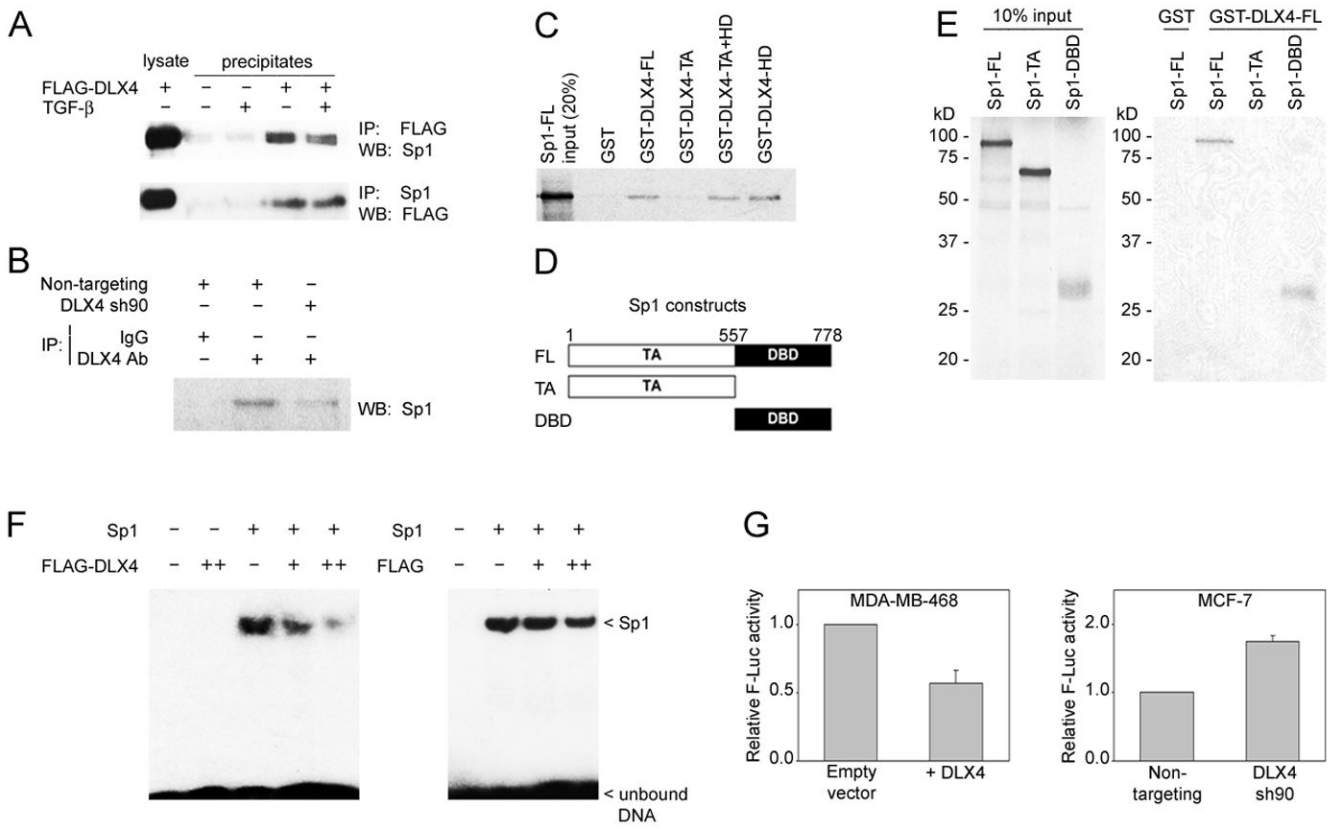

Figure 6.

DLX4 binds to the DNA-binding domain of Sp1. [A] Lysates were prepared from HepG2 cells as described in Figure 5A. Interaction of FLAG-DLX4 with Sp1 was detected by reciprocal IP using FLAG and Sp1 Abs. [B] MCF-7 cells were transfected with non-targeting shRNA or with *DLX4* (sh90) shRNA. Endogenous DLX4 was immunoprecipitated using DLX4 Ab, and precipitates analyzed by immunoblotting using Sp1 Ab. IP using mouse IgG was included as a negative control. [C] GST-DLX4 proteins (described in Figure 5D) were assayed for binding to ^{35}S-labeled full-length Sp1. [D] Sp1 constructs comprising the transactivation domain (TA) and DNA-binding domain (DBD). [E] ^{35}S-labeled full-length (FL) and truncated Sp1 were translated *in vitro (left)* and assayed for binding to full-length GST-DLX4 protein *(right)*. [F] Gel shift analysis using a ^{32}P-labeled oligonucleotide containing nucleotides −88 to −64 of the p15^{Ink4B} promoter (refer Figure 4D). Recombinant Sp1 protein was incubated with increasing amounts of *in vitro* translated FLAG-DLX4 and FLAG-tag. Gel-shifted DNA-bound Sp1 is indicated. [G] MDA-MB-468 cells were co-transfected with empty vector (-DLX4) or DLX4, together with the Cignal Sp1 reporter construct driven by a synthetic promoter comprising tandem Sp1-binding sites *(left)*. Sp1-driven promoter activity was likewise assayed in MCF-7 cells that were co-transfected with non-targeting shRNA or *DLX4* (sh90) shRNA *(right)*. Shown are average relative F-Luc activities in three independent experiments each performed in duplicate. Values were normalized by activity of co-transfected R-Luc.