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THE ROLE AND MECHANISM OF THE HOMEOBOX GENE DLX4 IN TRANSFORMING GROWTH FACTOR-B RESISTANCE IN CANCER

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THE ROLE AND MECHANISM OF THE HOMEOBOX GENE *DLX4* IN TRANSFORMING GROWTH FACTOR-β RESISTANCE IN CANCER

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Dedication

This dissertation is dedicated to my family for their love, encouragement and support, and to the memory of my beloved grandfathers

Thank you

Some parts of this thesis were modified from the following journal article:

Trinh BQ, Barengo N and, Naora H. Homeodomain protein DLX4 counteracts key transcriptional control mechanisms of the TGF-β cytostatic program and blocks the anti-proliferative effect of TGF-β. Oncogene. 2011, Feb 7. PMID: 21297662

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THE ROLE AND MECHANISM OF THE HOMEOBOX GENE *DLX4* IN TRANSFORMING GROWTH FACTOR-β RESISTANCE IN CANCER

Publication No._____

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Supervisory Professor: Honami Naora, Ph.D.

Transforming growth factor-β (TGF-β) is a cytokine that plays essential roles in regulating embryonic development and tissue homeostasis. In normal cells, TGF-β exerts an anti-proliferative effect. TGF-β inhibits cell growth by controlling a cytostatic program that includes activation of the cyclin-dependent kinase inhibitors $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ and repression of c-myc. In contrast to normal cells, many tumors are resistant to the anti-proliferative effect of TGF-β. In several types of tumors, particularly those of gastrointestinal origin, resistance to the anti-proliferative effect of TGF-β has been attributed to TGF-β receptor or Smad mutations. However, these mutations are absent from many other types of tumors that are resistant to TGF-β-mediated growth inhibition. The transcription factor encoded by the homeobox patterning gene *DLX4* is overexpressed in a wide range of malignancies. In this study, I demonstrated that DLX4 blocks the anti-proliferative effect of TGF-β by disabling key transcriptional control mechanisms of the TGF-β cytostatic program. Specifically, DLX4 blocked the ability of TGF-β to induce expression of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ by directly binding to Smad4 and to Sp1. Binding of DLX4 to Smad4 prevented Smad4 from forming transcriptional

complexes with Smad2 and Smad3, whereas binding of DLX4 to Sp1 inhibited DNA-binding activity of Sp1. In addition, DLX4 induced expression of c-myc, a repressor of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ transcription, independently of TGF-β 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. This study provides a molecular explanation as to why tumors are resistant to the anti-proliferative effect of TGF-β in the absence of mutations in the TGF-β signaling pathway. Furthermore, this study also provides insights into how aberrant activation of a developmental patterning gene promotes tumor pathogenesis.

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LIST OF ABBREVIATIONS

BMP	Bone morphogenetic protein
BRE	BMP response element
DMEM	Dulbecco's modified Eagle's medium
DBD	DNA-binding domain
EMT	Epithelial-Mesenchymal transition
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
F-Luc	Firefly luciferase
HDAC	Histone deacetylase
IP	Immunoprecipitation
I-Smad	Inhibitory Smad
MH1	Mad Homology 1
MH2	Mad Homology 2
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MEM	Minimum Essential Medium Eagle
MAPK	Mitogen-activated protein kinase
NLS	Nuclear localization signal
NES	Nuclear export signal
PBS	Phosphate buffered saline
qPCR	Quantitative PCR
Rb	Retinoblastoma protein

shRNA	small hairpin RNA
R-Smad	Receptor-regulated Smad
R-Luc	Renilla luciferase
SBE	Smad-binding element
shRNA	Short hairpin RNA
TGF-β	Transforming growth factor-β
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
TIE	TGF-β inhibitory element

CHAPTER 1: INTRODUCTION

A. TGF-β SIGNALING AND CANCER

Transforming growth factor-β (TGF-β) is a cytokine that controls diverse processes such as cell proliferation, differentiation and migration during normal embryonic development and in adult tissues (1, 2). TGF-β signaling has a complex role in tumors. On one hand, the anti-proliferative effect of TGF-β, which is essential for maintaining normal tissue homeostasis, is lost in many cancers. On the other hand, the ability of TGF-β to promote cell migration has an important role in tumor metastasis (3, 4).

1. The TGF super-family

The TGF super-family is comprised of more than 30 secreted proteins, including TGF-βs, activins and bone morphogenetic proteins (BMPs). These secreted proteins serve as ligands for specific transmembrane type I and type II receptors (3) (Table 1). Binding of the ligands to their specific type II receptor activates the corresponding type I receptor, which in turn activates specific receptor-activated Smad proteins (R-Smads) (Table 1). TGF-β and activin preferably activate Smad2 and Smad3, whereas Smad1, Smad5 and Smad8 are selectively activated by BMPs (5). Activation of R-Smads is inhibited by specific inhibitory Smad proteins (I-Smads) (i.e. Smad6, Smad7) (6, 7). All R-Smads, irrespective of their ligands, associate with a common Smad protein,

Table 1. The TGF super-family: Ligands, receptors and signal transducers

Major TGF ligands, their corresponding receptors and receptor-regulated Smads (R-Smads) are listed. All R-Smads interact with Smad4 as a common mediator (5, 9, 10).

<table>
<tr><th>Ligand</th><th>Type II receptor</th><th>Type I receptor</th><th>R-Smad</th></tr>
<tr><td>TGF-βs</td><td>TβRII</td><td>ALK5 (TβRI)</td><td rowspan="3">Smad2
Smad3</td></tr>
<tr><td rowspan="2">Activins
Nodals
Lefty</td><td>ActR-II</td><td>ALK4 (ActR-IB)</td></tr>
<tr><td>ActR-IIB</td><td>ALK7</td></tr>
<tr><td rowspan="3">BMPs</td><td>BMPR-II</td><td>ALK3 (BMPR-IA)</td><td rowspan="5">Smad1
Smad5
Smad8</td></tr>
<tr><td>ActR-II</td><td>ALK6 (BMPR-IB)</td></tr>
<tr><td>ActR-IIB</td><td rowspan="2">ALK2</td></tr>
<tr><td>MIS</td><td>MISRII</td></tr>
<tr><td>?</td><td>?</td><td>ALK1</td></tr>
</table>

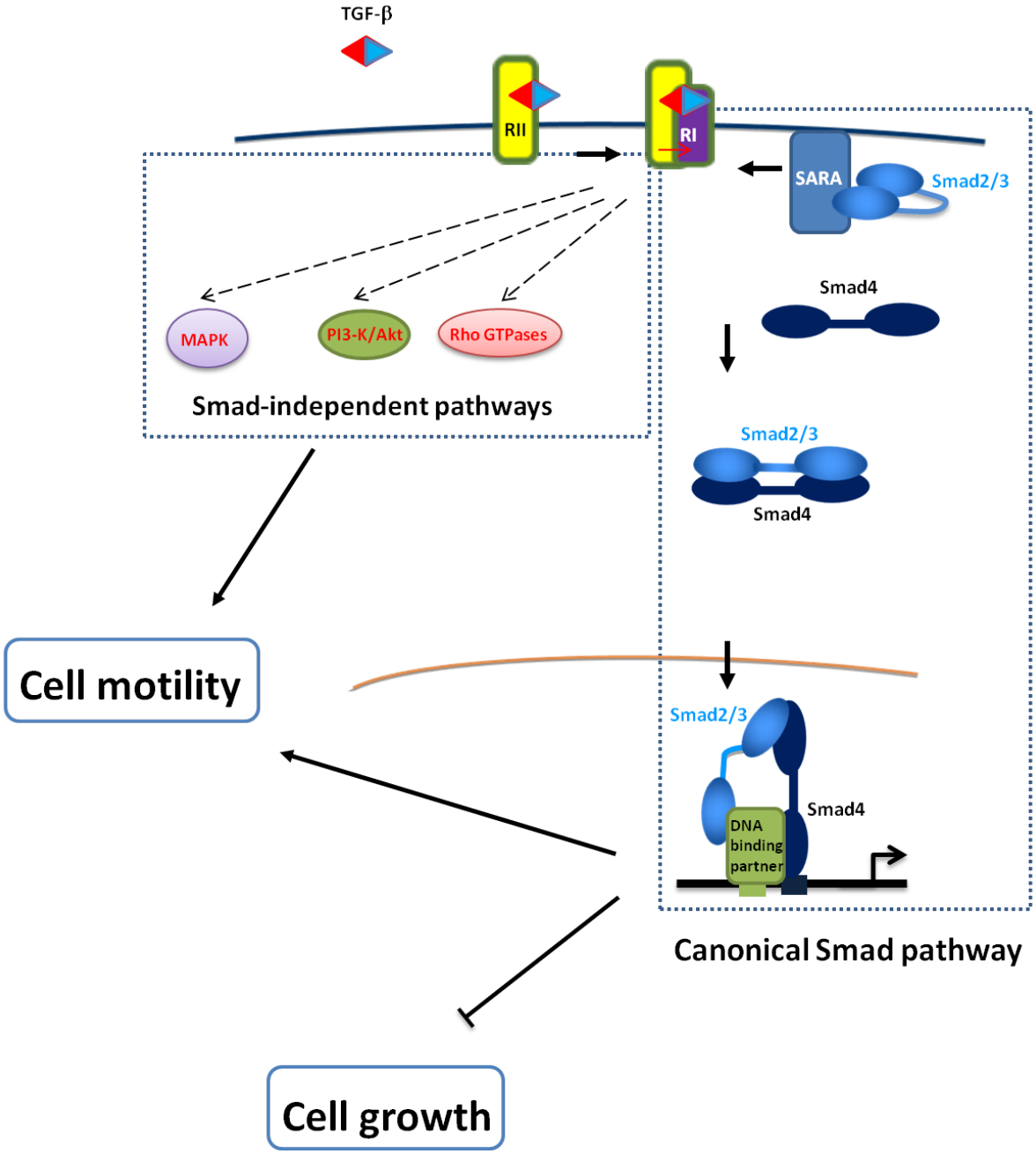
Smad4, to form heteromeric complexes in the nucleus that control transcription of distinct sets of genes (8).

1.1. TGF-β signaling pathways

Proteolytic cleavage of latent TGF-β releases a homodimeric complex of two polypeptides linked by disulphide bonds (bioactive TGF-β) from latent TGF-β binding proteins that tether the ligand in extracellular matrix (11). Binding of active TGF-β to the TGF-β type II receptor (TβRII) activates its serine/threonine kinase domain and recruits the TGF-β type I receptor (TβRI or ALK5). The TβRII kinase phosphorylates TβRI at several serine and threonine residues located within its juxtamembrane domain and thereby induces TβRI kinase activity (12). In turn, TβRI kinase phosphorylates Smad2 and Smad3 that are tethered to the receptor complex by the recruiting protein SARA. Phosphorylated Smad2 and Smad3 translocate into the nucleus, where they form heteromeric complexes with Smad4 and other DNA-binding factors to activate or repress transcription of distinct sets of genes (9, 12) (Figure 1). As discussed in more detail below, this canonical Smad-dependent pathway is crucial for mediating the anti-proliferative effect of TGF-β. Induction of cell migration by TGF-β is also controlled in part by the canonical Smad pathway (Figure 1). TGF-β can also trigger signaling pathways that are Smad-independent. Interaction of TβRII and/or TβRI with specific adaptor proteins activates distinct signaling pathways such as mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase/Akt (PI3-K/Akt) and Rho GTPases (13-16). These non-Smad signaling pathways are important for promoting cell migration in response to TGF-β (17) (Figure 1).

Figure 1. The TGF-β signaling pathways

TGF-β induces activation of Smad and non-Smad signaling pathways. TGF-β-mediated growth-inhibition is primarily controlled by the canonical Smad pathway. TGF-β-mediated cell motility is controlled by both Smad and non-Smad pathways. RII: TβRII, RI: TβRI.

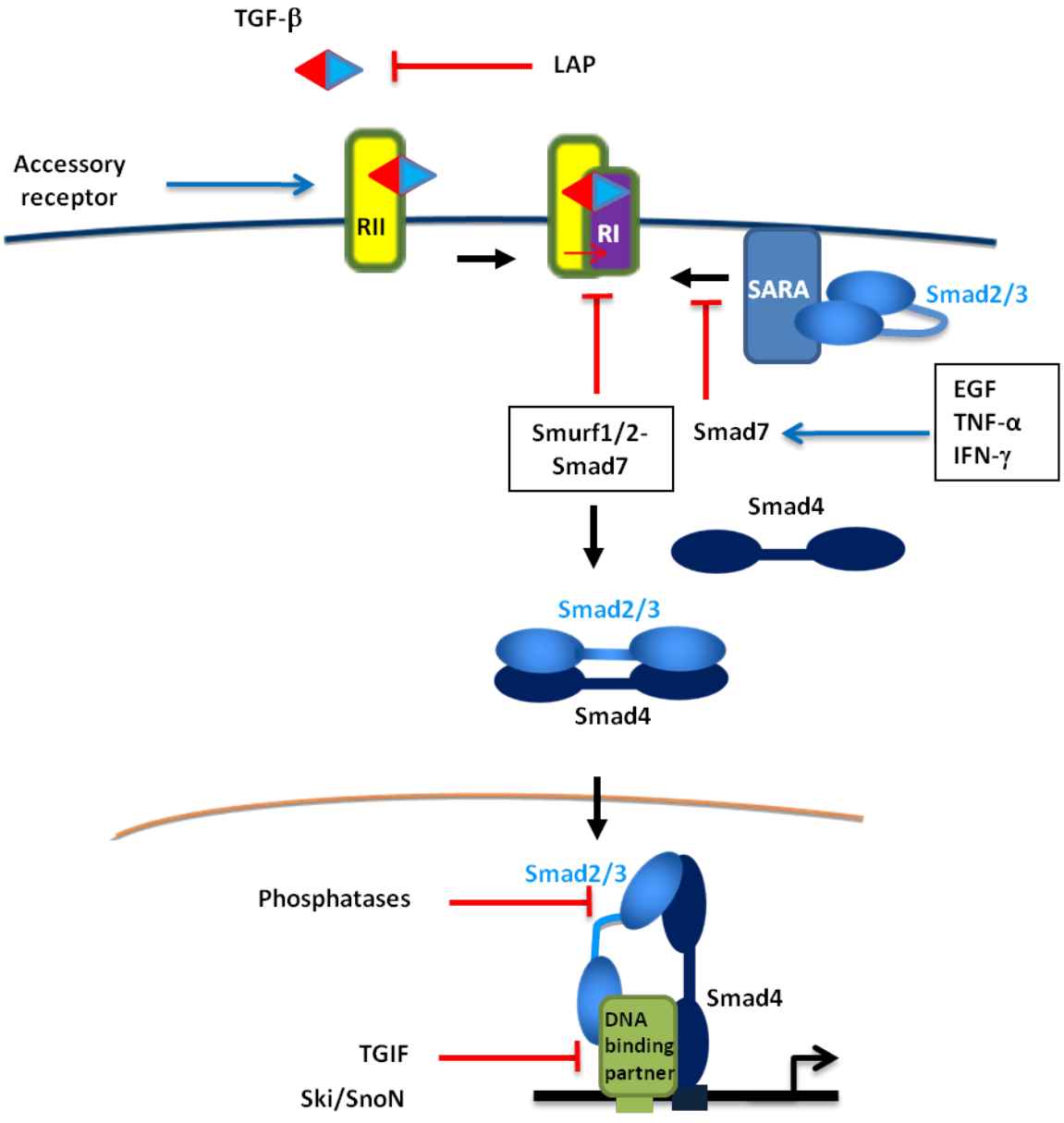


1.2. Modulation of TGF-β/Smad signaling

The Smad-dependent TGF-β signaling pathway is dynamically modulated at multiple levels, from ligand-binding to downstream nuclear events (Figure 2). Binding of TGF-β to TβRII is promoted by extracellular matrix proteins such as betaglycan, endoglyn and crypto, and is blocked by LAP protein (reviewed in (11)). Stability of the TβRI-TβRII complex is down-regulated by Smad7 that acts as an adaptor for the E3 ubiquitin ligases Smurf1 and Smurf2 (18, 19). Smad7 also prevents recruitment of R-Smads to TβRI (20). Signaling pathways triggered by various growth factors such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and epidermal growth factor (EGF) can indirectly inhibit TGF-β signaling by inducing expression of Smad7 (reviewed in (21)). Nuclear localization and activation of R-Smads are induced by TβRI phosphorylation, and are inhibited by phosphatases (22-24). Cytoplasmic retention of R-Smads is also caused by extracellular signal-regulated kinase (ERK)-mediated phosphorylation (25). TGF-β-induced Smad-dependent transcription is negatively modulated by several Smad co-repressors. For example, binding of transforming growth interacting factor (TGIF) to Smad2 represses transcription by recruiting histone deacetylases (HDACs) to the Smad transcriptional complex (26). Another example is the binding of Ski/SnoN to Smad transcriptional complexes that leads to recruitment of N-CoR/mSin3/HDAC repressor complexes (27-29).

Figure 2. Modulation of TGF-β/Smad signaling

TGF-β/Smad signaling is modulated at multiple levels from ligand-binding to downstream nuclear events. Several key "control-points" are indicated. Red lines represent inhibition; blue arrows represent induction.



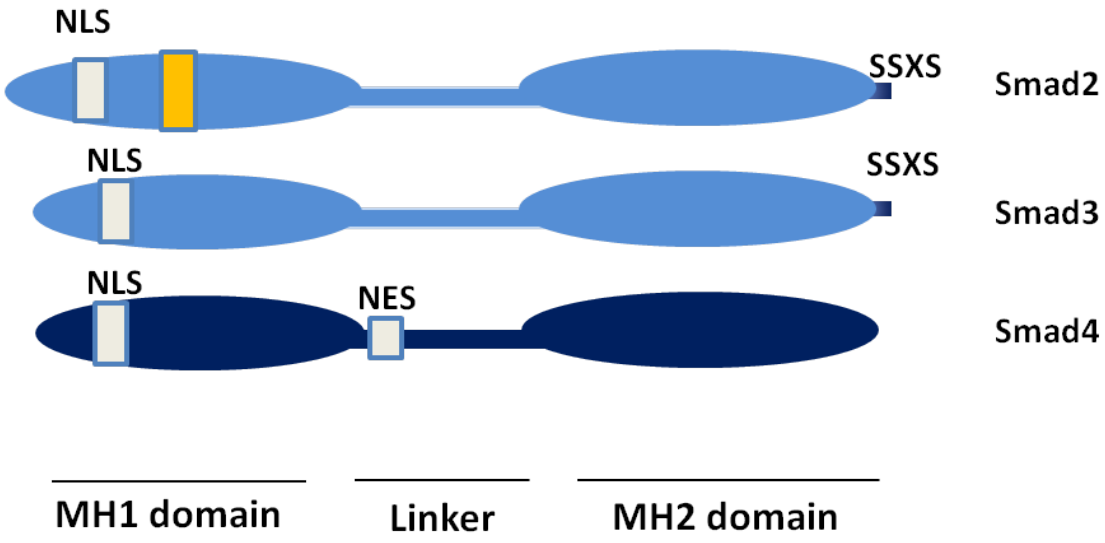
2. Smad-mediated transcription

2.1. Functional domains of Smad proteins

Smad proteins are comprised of two functional domains, Mad homology 1 (MH1) and Mad homology 2 (MH2), with an intervening linker region (8, 12) (Figure 3). MH1 domains contain nuclear localization signal (NLS) sequences and are responsible for interacting with Smad-binding DNA elements (SBE). MH2 domains are highly conserved between R-Smads. MH2 domains mediate interactions of R-Smads with TβRI, with one another, with Smad4, and with other transcription factors (12). Phosphorylation of two serine residues at the unique C-terminal Ser-Ser-X-Ser (SSXS) motif by TβRI kinase results in nuclear localization and activation of R-Smads (30, 31). Conversely, phosphorylation of linker domains by ERKs causes cytoplasmic retention of R-Smads. Linker domains are divergent among R-Smads and Smad4. The linker region of Smad4 possesses a nuclear export signal (NES) (Figure 3). This NES and the NLS signal, located in the MH1 domain, allow Smad4 to shuttle in and out of the nucleus (Figure 3).

Figure 3. Functional domains of Smad proteins

MH1 domains of Smad4 and R-Smads contain a nuclear localization signal (NLS). Smad4 also contains a nuclear export signal (NES). The MH1 domains mediate DNA binding. Smad2 contains an extra 30 amino acids (yellow box) that abrogates its DNA-binding ability. MH2 domains of R-Smads contain the SSXS motif that is phosphorylated by TβRII kinase. Adapted with permission from Wiley-Liss, Inc: JCB, Kimberly *et al*., copyright 2007 (32)



2.2. Smad-mediated transcription regulation

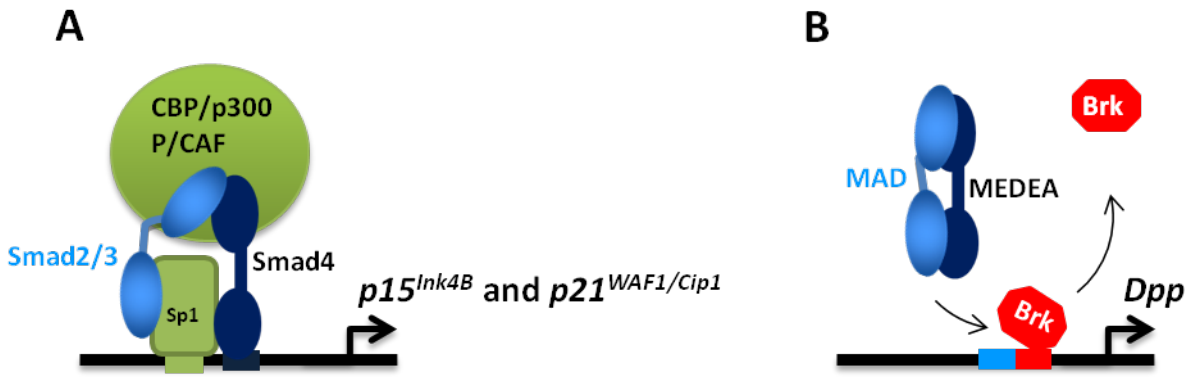
TGF-β induces formation of R-Smad/Smad4 complexes either as hetero-trimers (two R-Smads + Smad4) or as hetero-dimers (one R-Smad + Smad4) (33). The binding affinity of Smad MH1 domains to SBEs (5'-AGAC-3') is very low (33). However, high DNA-binding affinity and specificity are achieved by interaction of Smad hetero-dimers or -trimers with other DNA-binding factor(s) to form large transcriptional complexes that either induce or repress specific sets of target genes (8, 34, 35).

Smad-mediated transcription induction

Both the strength and specificity of Smad-induced transcription are governed by interactions between activated R-Smads, Smad4, and specific transcriptional activators with their corresponding DNA-binding elements on target promoters. A classic example is the induction by TGF-β of expression of the cyclin-dependent kinase (CDK) inhibitor $p15^{Ink4B}$. The proximal promoter region of the $p15^{Ink4B}$ gene (positions −110/−40) contains two adjacent sets of SBEs and GC boxes that are bound by the transcription factor Sp1 (36). TGF-β induces $p15^{Ink4B}$ transcription by stimulating cooperative interactions between Sp1 and Smad proteins that enhance their DNA-binding and transcriptional activities (Figure 4A). Transcriptional activation by TGF-β of the gene encoding the CDK inhibitor $p21^{WAF1/Cip1}$ is also mediated by cooperative interactions between Sp1 and Smads. This is achieved via two distinct promoter regions, a distal region (located 1.7 kb upstream of the transcription start site) that contain SBEs, and a proximal region (located between positions −124 to −61)

Figure 4. Smad-mediated transcriptional activation

Smads can induce transcription of target genes by **[A]** recruiting transcriptional activators (e.g. Sp1) and/or histone acetylases (e.g. CBP/p300) and by **[B]** displacing transcriptional repressors (e.g. Brk).



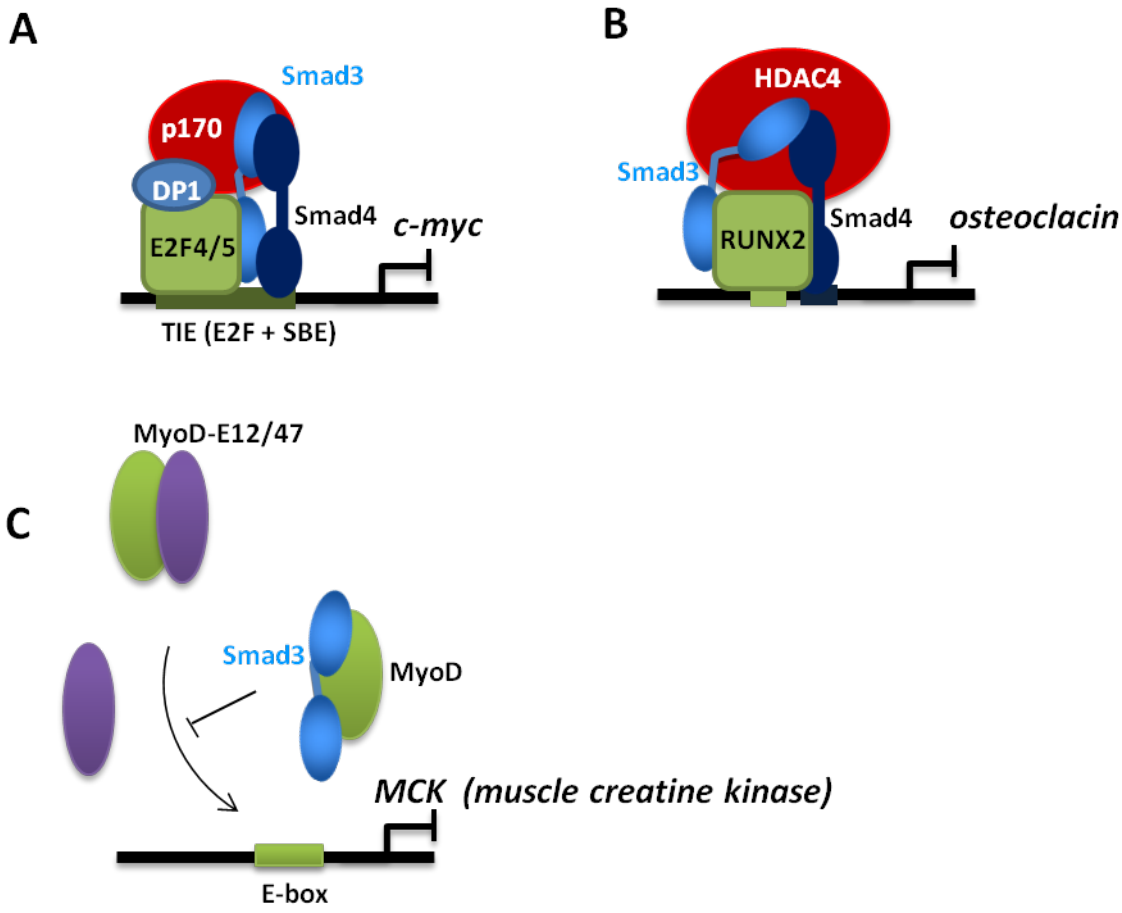
that have Sp1-binding sites (37, 38). It is thought that coupling of the distal and proximal promoter elements and formation of the Smad-Sp1 transcription complex occurs via chromatin remodeling (10, 34, 39). Increasing evidence indicates that the histone acetyltransferase CBP/p300 is a major co-activator for Smad-mediated transcriptional activation (40-43) (Figure 4A). Smads also can activate specific genes by displacing transcriptional repressors. For example, binding of MAD (*Drosophila* homolog of R-Smad) to the *Dpp (Decapentaplegic)* promoter dislodges the Brk (Brinker) repressor from the *Dpp* promoter (44) (Figure 4B).

Smad-mediated transcription repression

Although Smad proteins have intrinsic trans-activating activity, gene repression accounts for about one-quarter of TGF-β-mediated gene responses (45). Mounting evidence implicates a central role for Smad3, but not Smad2, in TGF-β-mediated transcription repression. A well-studied example is TGF-β-mediated repression of c-*myc* transcription (Figure 5A). Smad3 binds to the co-repressor p107 in a complex with E2F4/5 and DP1. In response to TGF-β stimulation, this complex translocates into the nucleus and interacts with Smad4 via Smad3. Smad4 binds to the SBE, while E2F4/5 binds to the E2F binding site. The E2F binding site and the SBE are both located within the TGF-β inhibitory element (TIE) of the c-*myc* promoter. Interaction of E2F4/5-DP1-p107-Smad3-Smad4 repressive complex with the TIE element blocks c-*myc* transcription (46, 47) (Figure 5A). On the other hand, *Id1* transcription is blocked by the Smad co-repressor ATF3. ATF3 forms inhibitory complexes with Smad3 and Smad4, and ATF3 expression itself is induced by Smads (48). Smad3 also represses

Figure 5. Smad-mediated transcriptional repression

Smads can repress transcription of target genes by **[A]** recruiting transcriptional repressors (e.g. p107), **[B]** recruiting HDACs (e.g. HDAC4), and **[C]** sequestering transcriptional activators (e.g. MyoD).



transcription by recruiting HDACs. Smad3 blocks osteoclacin expression by binding Runx2 and recruiting HDAC4 to form a repressive complex that inhibits Runx2-induced *osteoclacin* transcription (49) (Figure 5B). In addition to recruiting repressors, Smad3 blocks binding of transcriptional activators to target gene promoters. For example, Smad3 inhibits transcription of the muscle creatine kinase (*MCK*) gene by binding MyoD and blocking formation of MyoD-E12/47 dimers and their binding to the E-box motif (50) (Figure 5C).

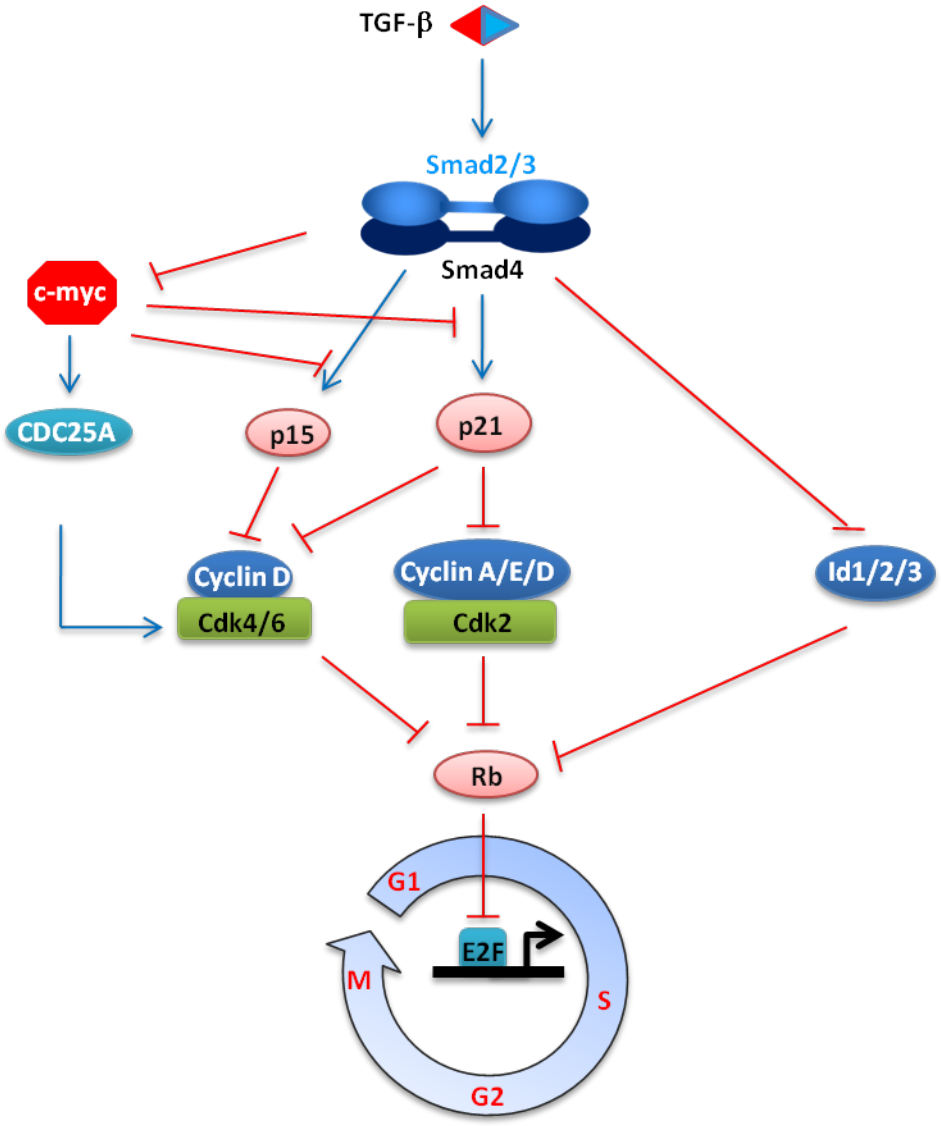
3. Cellular responses to TGF-β

3.1. TGF-β-mediated growth inhibition

In most types of normal cells, TGF-β has a potent anti-proliferative effect. TGF-β primarily inhibits cell growth by inducing cell cycle arrest in G_1 phase (3). TGF-β controls a cytostatic program of gene responses that includes activation of CDK inhibitors and repression of growth-promoting transcription factors (Figure 6). Gene responses that are central to this program are induction of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ (38, 51) and repression of c-*myc* and inhibitors of differentiation (*Ids)* (3, 48, 52). Each of these gene responses leads to G_1 arrest by distinct but integrated mechanisms. $p15^{Ink4B}$ forms an inactive complex with CDK4/6 that prevents activation of CDK4/6 by cyclin D (53) (Figure 6). Conversely, $p21^{WAF1/Cip1}$ binds to and inhibits cyclin A/D/E-CDK2 and cyclin D-CDK4/6 complexes (54) (Figure 6). E2F family members are crucial for G_1/S transition and S phase progression (55). Inhibition of CDKs keeps the Rb protein in an unphosphorylated and active form such that Rb is able to bind and

Figure 6. TGF-β induces cell cycle arrest

TGF-β induces expression of the CDK inhibitors, $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, and inhibits expression of c-myc and Id transcription factors. Induction of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ and down-regulation of c-myc and Ids lead to increased repression by Rb protein of the E2F transcription factors that normally promote G_1 to S phase progression. Red lines represent inhibition; blue arrows represent induction.

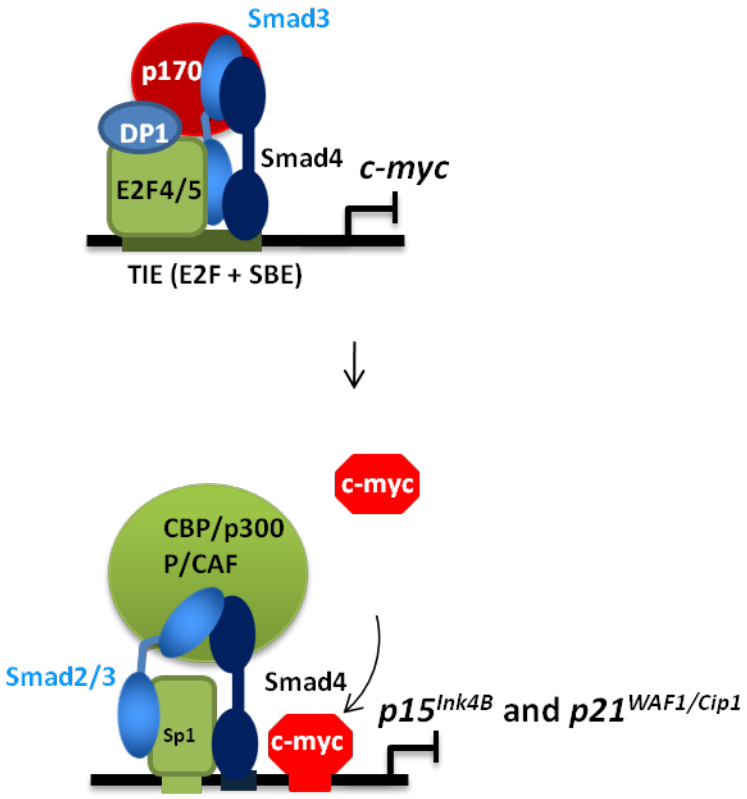


block transcriptional activity of E2F proteins (56) (Figure 6). On the other hand, c-myc induces expression of cdc25A which dephosphorylates and activates Cdk4/6 (57) (Figure 6). TGF-β also inhibits expression of Id1, Id2 and Id3 (3, 48, 58). Binding of Id proteins to unphosphorylated Rb dislodges Rb from E2F transcription factors, thereby releasing E2Fs from the inhibitory effect of Rb (59) (Figure 6). As discussed above (section 2), transcriptional activation of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, and repression of c-*myc* and *Ids* occur in a Smad-dependent manner (Figures 4, 5). However, there is evidence that TGF-β can also induce G_1 arrest by a Smad-independent mechanism. It has been reported that TGF-β induces protein phosphatase 2A (PP2A) that dephosphorylates and deactivates p70S6K, a kinase that plays a role in G_1/S progression (60, 61). The TGF-β cytostatic program is tightly integrated by feedback loops that protect against competing mitogenic signals. c-myc represses transcription of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, but its own transcription is repressed by TGF-β (46, 62, 63) (Figure 7). TGF-β/Smad signaling inhibits *Id2* expression by repressing expression of c-myc, an activator of *Id2* transcription, and by inducing expression of the c-myc antagonist MAD4 (58, 64).

The role of TGF-β in programmed cell death varies depending on the cell type and cellular conditions (65-69). Several genes that are induced in a Smad-dependent manner can promote apoptosis. For example, TIEG, an early response gene induced by TGF-β, induces apoptosis in pancreatic epithelial cell lines (68). On the other hand, activation of several Smad-independent pathways by TGF-β can inhibit apoptosis. TGF-β blocks serum withdrawal-induced apoptosis by suppressing JNK activity in lung

Figure 7. Integrated control of the TGF-β cytostatic program

The TGF-β cytostatic program is tightly integrated by feedback loops that protect against competing mitogenic signals. For example, c-myc represses transcription of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$. On the other hand, c-*myc* transcription is repressed by TGF-β .



carcinoma cells and Akt activity in mammary epithelial cells and skin keratinocytes (65, 67). However, the program of TGF-β-mediated apoptosis is not well-defined (3)

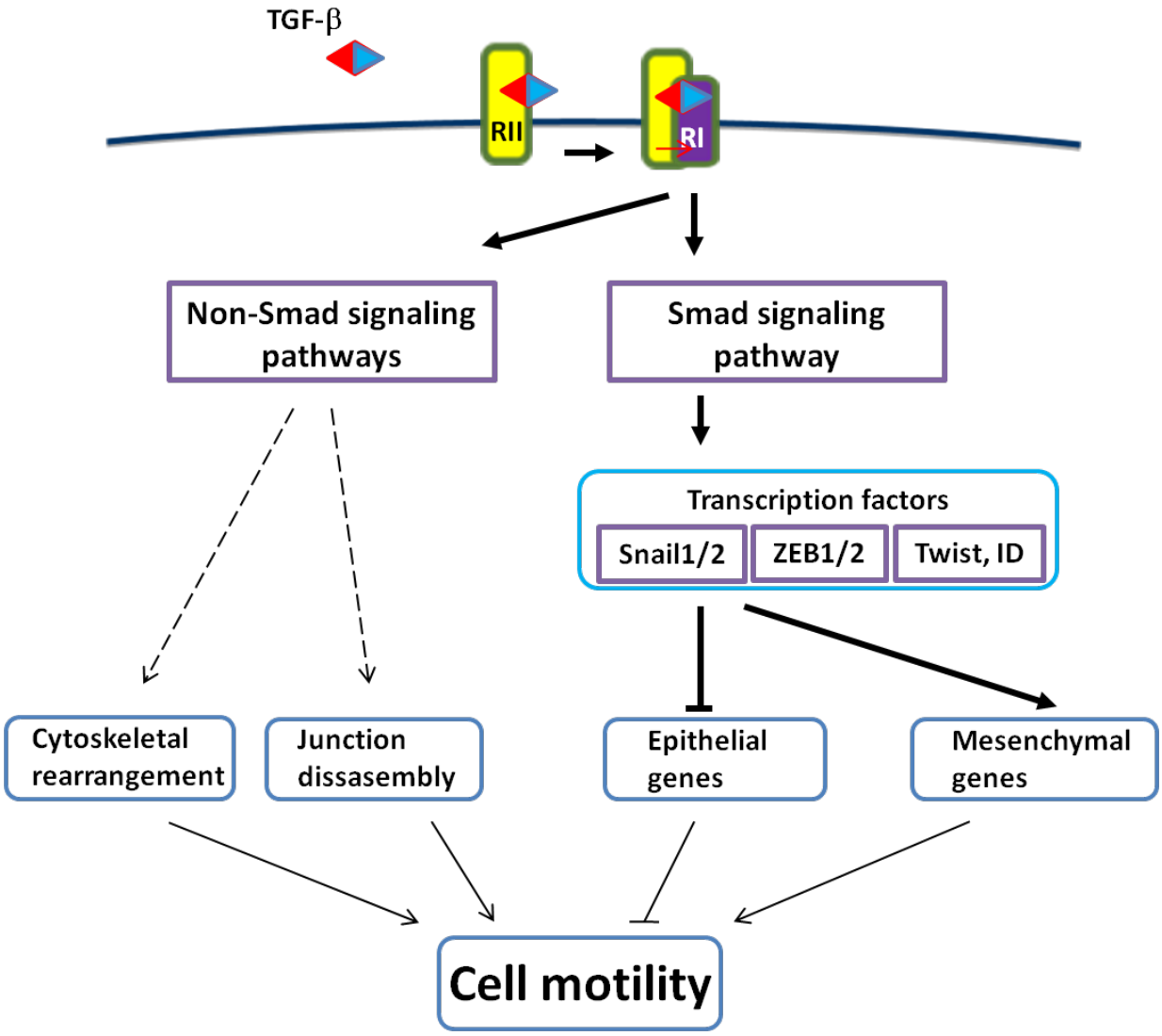
3.2. TGF-β-induced cell motility

During normal developmental patterning and tissue repair, cells often acquire migratory and invasive properties (2). In response to TGF-β, epithelial cells acquire fibroblastic morphology and become motile and invasive. The hallmarks of this conversion, termed epithelial-to-mesenchymal transition (EMT), are cytoskeletal reorganization (actin reorganization, stress fiber formation), junctional disassembly (dissolution of tight junctions, adherens junctions and desmosomes) and loss of apical-basolateral polarity (2, 17).

TGF-β promotes EMT in part by inducing expression of transcription regulators that belong to the Snail, ZEB and bHLH families in a Smad-dependent manner (Figure 8). These transcription factors repress expression of major transmembrane components of adherens and tight junctions of epithelial cells, and conversely activate expression of mesenchymal genes (2). Snail1 and Snail2 (Slug) repress expression of E-cadherin, plakoglobin, claudins and occludin (70-72). Conversely, Snail proteins induce expression of vimentin, fibronectin, vitronectin and N-cadherin (73, 74), extracellular matrix proteins (collagen type III and V) (73) and regulators of migration and invasion (RhoB, plasminogen activator inhibitor-1, matrix metalloproteinase MMP-9) (74, 75). ZEB1 and ZEB2 (SIP1) repress expression of E-cadherin (76, 77), desmosome protein plakophilin-2 and tight junction proteins claudin-4 and ZO-3, and

Figure 8. Induction of EMT and cell motility by TGF-β

TGF-β promotes EMT and cell motility by both Smad-dependent and Smad-independent (non-Smad) mechanisms. EMT-inducing transcription factors (Snail, ZEB and bHLH families) are induced by TGF-β in a Smad-dependent manner. These transcription factors repress expression of epithelial genes and activate expression of mesenchymal genes. Activation of non-Smad signaling pathways by TGF-β enhances cell motility by inducing cytoskeletal reorganization and junctional disassembly.



conversely enhance expression of vimentin, N-cadherin and MMP-2 (78). The bHLH transcription factor members Twist1 and Twist2 repress expression of E-cadherin, occludin and claudin-7, and induce vimentin and N-cadherin (79).

Increasing studies indicate the importance of non-Smad signaling pathways in TGF-β-induced EMT (Figure 1). Inhibition of the MAPK, Rho GTPase and PI3-K/Akt pathways blocks TGF-β-induced EMT in epithelial cells (13, 80-83). Activation of MAPK members is mediated via different TGF-β receptor adaptor proteins. TRAF6 activates TAK1 which in turn activates p38 MAPK and c-Jun N-terminal Kinase (JNK) (84, 85). Shc activates Ras which then activates ERK1 and ERK2 (86). ERK1/2 indirectly regulate genes that control cell-matrix interactions, cell motility and endocytosis (45). Activation of MAPK signaling induces junction disassembly and cell motility (reviewed in Ref. (87, 88)). TGF-β induces rapid activation of Rho GTPases. Activated RhoA induces p160ROCK (RhoA kinase) (89) which induces stress fiber formation and stimulates LIM kinase. Induction of cofilin by LIM kinase causes re-organization of the actin cytoskeleton (90, 91). Conversely, RhoA activity is controlled by Par6 that activates the E3 ubiquitin ligase Smurf1, which in turn controls RhoA activity at tight junctions by targeting it for degradation (14). TGF-β induces interaction of the regulatory subunit of PI3-K with TβRI receptor resulting in rapid activation of the PI3-K/Akt signaling pathway (16). Activation of this signaling leads to reduced cell-cell adhesion and the acquisition of spindle cell morphology (13, 92, 93). Akt has been reported to repress transcription of E-cadherin (93), induce expression of metalloproteinases (94) and induce delocalization of E-cadherin, ZO-1 and integrin-β1 from cell junctions (13).

4. Complex role of TGF-β in tumorigenesis

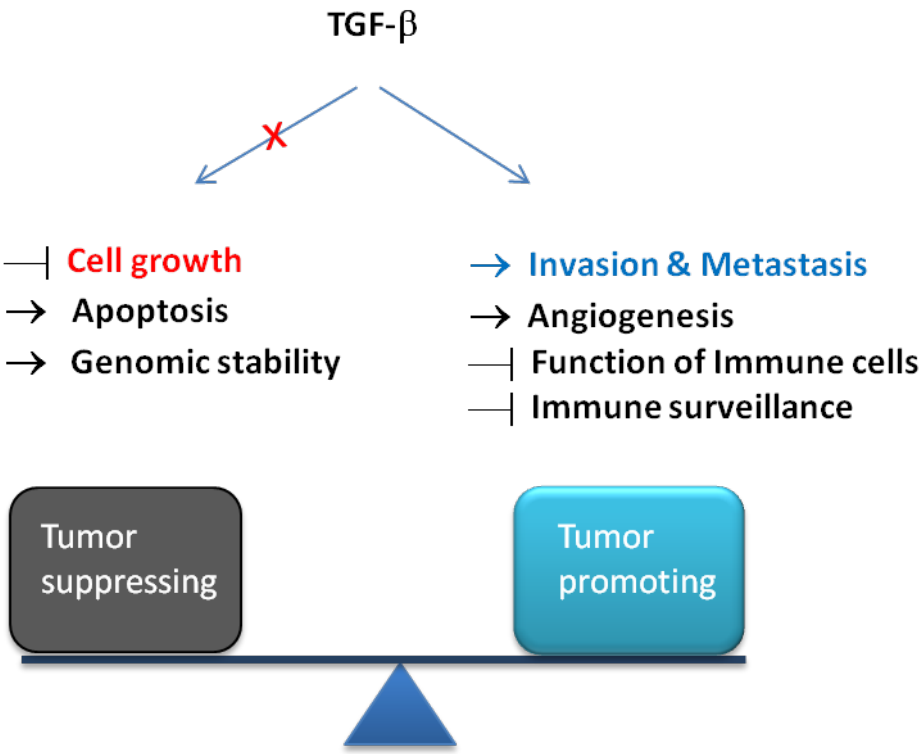
Tumorigenesis is a dynamic process characterized by multiple acquired capabilities that include insensitivity to anti-growth signals, limitless replicative potential, self-sufficiency in growth signals, evasion of apoptosis, sustained angiogenesis and tissue invasion (95). TGF-β plays a complex role in tumorigenesis by facilitating the cancer cell's acquisition of these key capabilities (21, 95) (Figure 9).

4.1. Promotion of tumor progression by TGF-β

TGF-β promotes tumor progression by exerting both cell-autonomous and non-cell-autonomous effects. The cell-autonomous effect of TGF-β is the recapitulation of normal TGF-β-induced EMT and is crucial for tumor metastasis (2, 9). Blockade of TGF-β signaling inhibits tumor cell invasiveness, whereas restoring TGF-β signaling in non-invasive tumor cells promotes invasiveness (96). TGF-β also promotes tumor progression by exerting non-cell-autonomous effects on the tumor microenvironment. TGF-β promotes tumor angiogenesis in part by inducing expression of angiogenic cytokines, and also MMP-2 and MMP-9 that induce endothelial cell migration and invasion (97-100). Furthermore, TGF-β causes immunosuppression and enables tumor cells to escape from immunosurveillance (101-103). TGF-β represses the function of cytotoxic T cells by inhibiting expression of interleukin-2 and its receptors, which are crucial for T-cell proliferation (104, 105). TGF-β causes loss of tumor cell immunogenicity by repressing expression of major histocompatibility complex antigens

Figure 9: Multiple roles of TGF-β in tumorigenesis

TGF-β signaling has a complex role in tumorigenesis. TGF-β induces migratory behavior of normal cells and promotes metastasis of tumor cells. TGF-β also promotes tumor angiogenesis and immunosuppression. TGF-β inhibits proliferation, induces apoptosis and maintains genomic stability in normal cells. The anti-proliferative effect of TGF-β is lost in many cancers.



(106-108). TGF-β also inhibits activity of other immune cells such as killer cells, NK cells (109), neutrophils (110), macrophages and B cells (102, 111).

4.2. Resistance of tumors to the anti-proliferative effect of TGF-β

A major hallmark of cancer is acquired insensitivity to anti-growth signals (95). In many normal cell types, TGF-β has a potent anti-proliferative effect. However, many tumors are resistant to the anti-proliferative effect of TGF-β (3, 4, 39). Mutations of some core components of the TGF-β signaling pathway such as TβRI, Smad2 and Smad3 are rarely found (<5%) in cancers (39, 112). However, resistance to the growth-inhibitory effect of TGF-β in some cancers has been attributed to mutations in other core components, in particular, TβRII and Smad4 (Table 2). Inactivation of TβRII due to somatic mutations has been reported to occur at high frequency (60-90%) in colon cancers with microsatellite instability (MSI) (112-114). Mutations or deletions of Smad4 have been reported in ~50% of pancreatic and non-MSI colorectal cancers (112, 115). TGF-β receptor mutations have been found in 12-31% of ovarian cancers (116, 117) but many ovarian cancers that are resistant to TGF-β were reported to express functional receptors (118). However, mutations in TβRII and Smad4 are rarely found in various other types of cancers such as lung and prostate cancers (112, 119-121) (Table 2). Similarly, many TGF-β-resistant breast cancers rarely contain TGF-βRII or Smad mutations (122, 123) (Table 2). The rareness of mutations in core components of the TGF-β signaling pathway in many tumors therefore indicates that resistance to TGF-β also stems from other aberrations.

Table 2. Mutation frequency of TGF-β signaling components in cancers

Mutation frequency of TβRII and Smad4 genes in some types of cancers are listed. +MSI: Cancer with microsatellite instability, -MSI: Cancer with no microsatellite instability. nd: Not detected. Mutations which are rarely detected are indicated as <5%:

Cancer	TβRII Gene	Smad4 Gene	Reference
Colon	60-90% (+MSI)	50% (-MSI)	(113-115, 124, 125)
Pancreas	4%	50%	(125)
Ovary	12-31%	12%	(116, 117)
Lung	nd (+MSI)	7%	(119, 121)
Prostate	<5%	<5%	(120)
Breast	<5%	<5%	(122, 123)

B. ABERRATIONS OF HOMEOBOX GENES IN HUMAN CANCERS

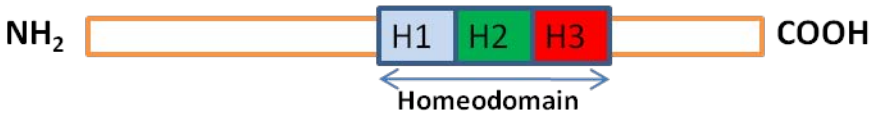
Homeobox genes comprise a large super-family of evolutionarily conserved genes that encode transcription factors (126, 127). Homeobox genes play essential roles in controlling developmental patterning (127-130). Many homeobox genes are aberrantly expressed in tumors, but the functional significance of their aberrant expression is poorly understood (131).

1. Overview of homeobox genes

Transcription factors encoded by homeobox genes are characterized by their highly conserved 61 amino acid DNA-binding domain termed the homeodomain. This domain forms three alpha helices that bind DNA elements containing a TAAT core motif (127) (Figure 10). The homeobox gene super-family is categorized into several different families based on sequence similarities in their homeodomain and other functional motifs (132, 133) (Figure 11). Members of the *HOX* and *DLX* homeobox gene families are organized in clusters. In mammals, the 39 members of the *HOX* family are organized in four clusters located on different chromosomes. The 6 members of the *DLX* family are located upstream of these *HOX* loci (134, 135) (Figure 12). These *HOX-DLX* clusters have been postulated to derive from gene duplication during evolution (128, 136).

Figure 10. Structure of homeodomain proteins

Transcription factors encoded by homeobox genes contain a conserved homeodomain that binds DNA. The homeodomain forms three α-helixes (colored boxes) that bind DNA sequences containing a TAAT core motif. Adapted with permission from Nat Rev Cancer, Abate-Shen, copyright 2002 (131).



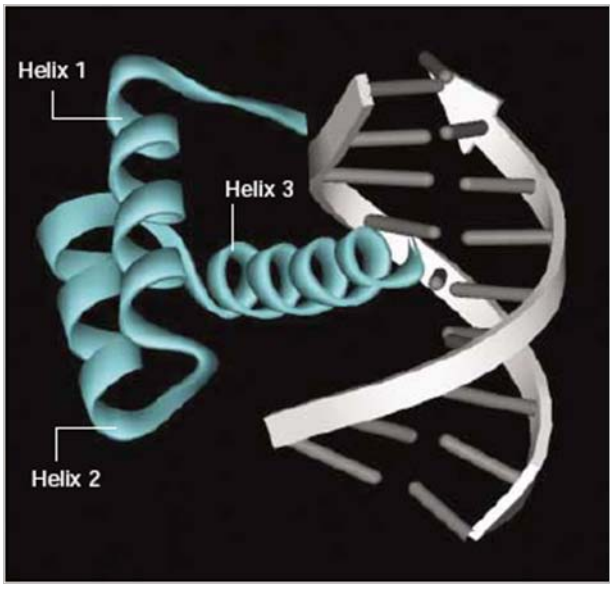


Figure 11. Classification of homeobox genes

The homeobox gene super-family comprises more than 200 genes that are categorized into different families. The *DLX* family comprises six members.

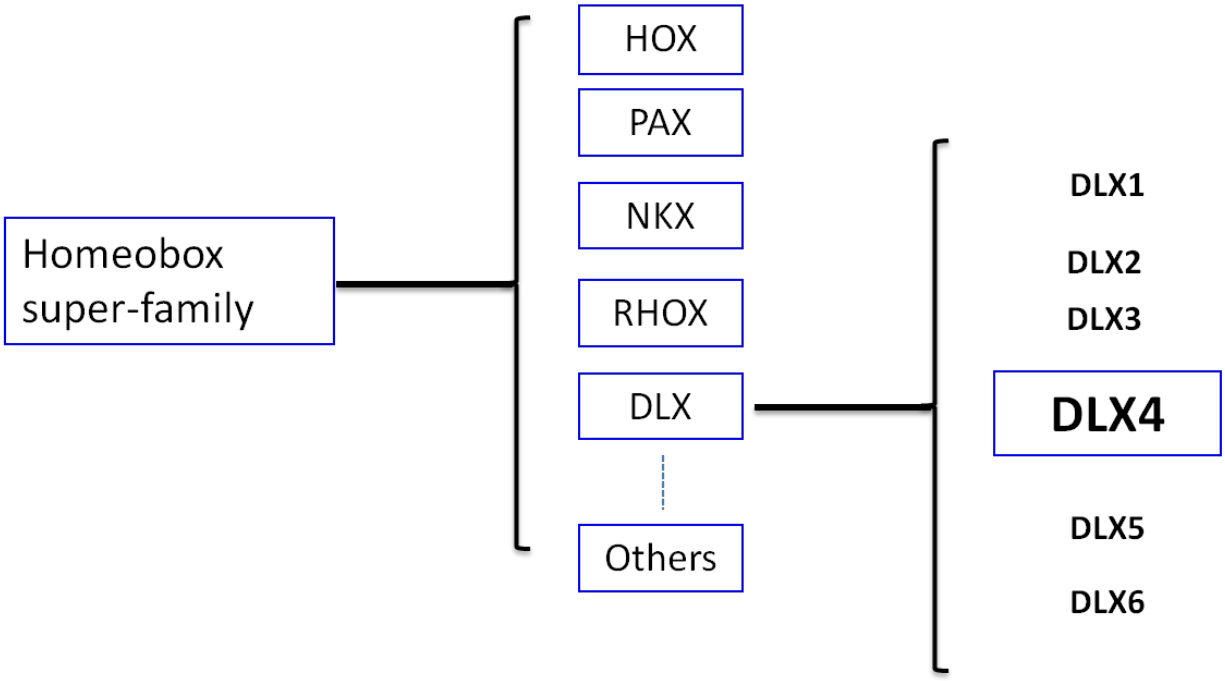
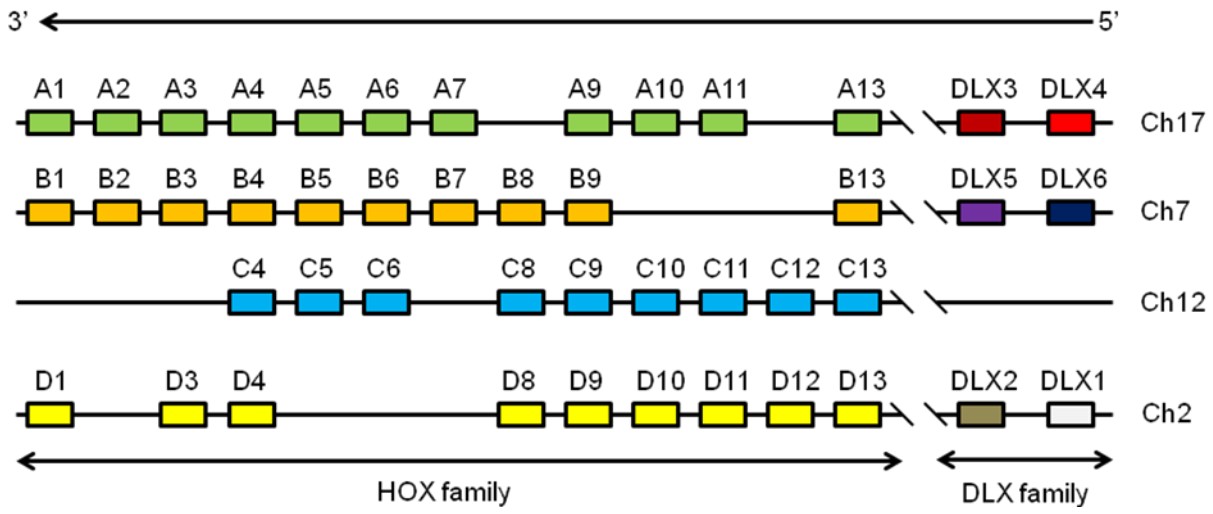


Figure 12. Gene clusters of *DLX* and *HOX* families

Members of the mammalian *HOX* and *DLX* homeobox gene families are organized in clusters. The 39 members of the *HOX* family are organized in four clusters located on different chromosomes. The 6 members of the *DLX* family are located upstream of these *HOX* loci. Adapted with permission from Springer: Cell, Krumlauf, copyright 1994 (128).



2. Homeobox genes in development and cancer

Homeobox genes play essential roles in controlling cell differentiation, tissue morphogenesis and specification of the body plan during embryonic development. Distinct sets of homeobox genes control organogenesis, development of the central nervous system, limb and skeletal patterning, and craniofacial morphogenesis (137, 138). Homeobox genes also control cell renewal and tissue regeneration processes in adults, including hematopoiesis, spermatogenesis and endometrial remodeling during the menstrual cycle (139-143). Aberrant expression of various homeobox genes has been reported in different types of tumors (Tables 3, 4). A general trend is that homeobox genes that are normally expressed in differentiated adult tissues are down-regulated in tumors, whereas homeobox genes that are normally expressed in embryonic tissues are activated in tumors (131, 144) (Figure 13). This aberrant expression of homeobox genes in tumors is thought to reflect an inappropriate recapitulation of embryonic pathways (131, 144). Several homeobox genes that are down-regulated in tumors have tumor-suppressive functions (144) (Table 3). Loss of function of these homeobox genes appears to drive cells back to a less differentiated state and promotes cell survival and proliferation (144). Down-regulated expression of several homeobox genes in tumors has been attributed to epigenetic mechanisms and chromosomal aberrations. For example, loss of *HOXA5* expression in breast tumors is due to promoter methylation (145). Loss of *NKX3.1* and *CUTL1* expression in prostate and uterine cancers stems from loss of heterozygosity (146, 147). On the other hand, aberrant activation of several homeobox genes that are normally expressed in embryonic tissues promotes tumor cell proliferation and survival (Table 4). Aberrant expression of homeobox genes in leukemias primarily arises from chromosomal

Table 3. Examples of homeobox genes that are down-regulated in tumors

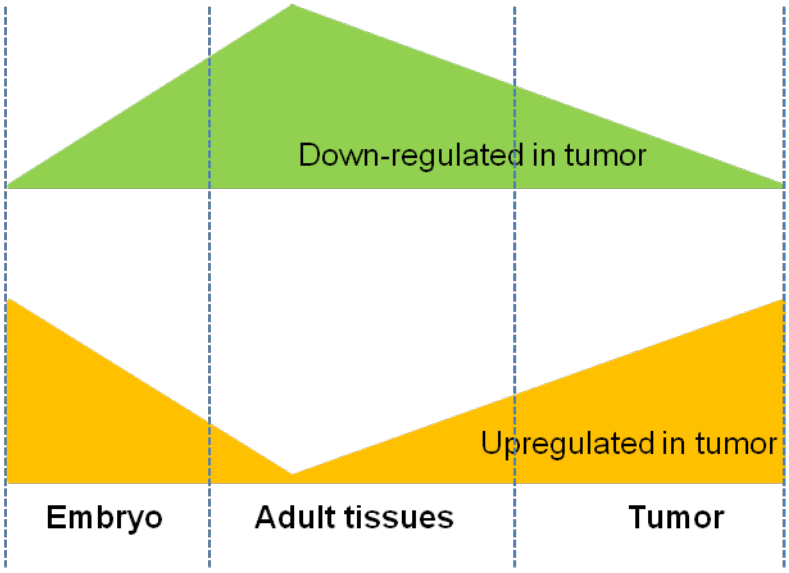
Gene	*Cancer* (↓)	Normal expression pattern	Functional significance	Reference
CDX2	Colon (↓)	Expressed in adult intestinal epithelium	Inhibits growth of colon carcinoma cells by inducing expression of $p21^{WAF1/Cip1}$. Heterozygous deletion increases susceptibility to carcinogenesis.	(149-152)
NKX3.1	Prostate (↓)	Expressed in adult prostate epithelium	Null mutant mice are predisposed to prostate cancer. Loss of expression correlates with tumor progression.	(146, 153, 154)
HOXA5	Breast (↓)	Expressed in normal breast epithelium.	Regulate p53 expression .Loss of expression was found in 60% of breast cancer to be due to promoter methylation.	(145, 155)
BARX2	Ovary (↓)	Expressed in normal adult ovarian surface epithelium	Has tumor suppressive and anti-metastatic properties. Can modulate cisplatin sensitivity. Frequently deleted in ovarian cancer	(156, 157)

Table 4. Examples of homeobox genes that are up-regulated in tumors

Gene	*Cancer* (↑)	Normal expression pattern	Expression and Function in tumors	Reference
PAX2	Renal (↑)	Expressed during embryonic urogenital development but not in adult kidney.	Expressed in renal cell carcinomas. Promotes cancer cell survival.	(158-161)
HSIX1	Breast (↑)	Expressed in developing eye, brain, muscle, embryonic mammary gland.	Promotes cell cycle progression by activating cyclin A1 expression.	(162, 163)
GBX2	Prostate (↑)	Expressed in developing nervous system.	Induces cell growth by inducing expression of interleukin-6.	(164, 165)
HOXB7	Melanoma Ovarian Breast		Promotes tumor growth and angiogenesis by inducing expression of fibroblast growth factor-2. Induces EMT. Promotes DNA repair.	(166-169)

Figure 13. Aberrant expression of homeobox genes in tumors

Homeobox genes that are expressed during embryonic development, but are down-regulated in normal adult tissues, are often up-regulated in tumors. Conversely, homeobox genes that are expressed in normal adult tissues are often down-regulated in tumors. Adapted with permission from Nat Rev Cancer, Abate-Shen, copyright 2002 (131).



translocations. Several chimeric oncoproteins arise from fusion of the *NUP98* gene and *HOX* genes (144, 148). However, the mechanisms that give rise to activation of homeobox genes in solid tumors are largely unknown.

3. Significance of the homeobox gene *DLX4* in cancer

Most homeobox genes are expressed in a tissue-specific manner. *DLX4*, a member of the *DLX* homeobox gene family, is expressed in normal bone marrow cells, trophoblast, placenta and endometrium, but is not expressed in most other normal adult tissues (170-172). *DLX4* has been increasingly reported to be expressed in diverse types of tumors (Table 5). The *DLX4* gene maps to the 17q21.3-q22 region (173, 174). Amplification of this chromosomal hot-spot in breast and ovarian cancers correlates with poor prognosis (175). Aberrant expression of *DLX4* in ovarian cancers is significantly associated with high tumor grade and advanced disease stage (173). Aberrant expression of *DLX4* in breast cancers correlates with invasiveness (176). Upregulation of *DLX4* has also been reported in other types of cancers, including leukemia, choriocarcinoma, prostate and lung cancers (172, 177-179). The upregulation of *DLX4* in tumors arising from a wide variety of organ sites raises the strong possibility that *DLX4* controls a pathogenic mechanism that is common to multiple types of tumors.

Table 5. Aberrant expression of DLX4 in tumors

Cancer	Expression pattern in tumors	Functional significance	Reference
Ovarian	Expressed in ovarian carcinomas and correlates with high tumor grade and disease stage.	Induces tumor vascularization by inducing expression of VEGF and FGF-2.	(173)
Breast	Highly expressed in invasive tumors.	Represses expression of BRCA1. Induces expression of bcl-2 and inhibits TNF-α-induced apoptosis in breast cancer cells.	(176, 187-190)
Leukemia	Highly expressed in chronic lymphocytic leukemia and acute lymphoblastic leukemia.	Expression in leukemic cells increases clonogenicity.	(172)
Prostate	Expressed in 70% of prostatic adenocarcinomas.		(177)
Lung	Expressed at higher levels in tumors than in adjacent normal tissues.	Enforced expression of DLX4 in metastatic lung cancer cells inhibits metastasis.	(178, 179)
Chorio-carcinoma	Expressed in placenta and choriocarcinoma cell lines	Promotes tumor cell survival	(191, 192)

C. CROSS-REGULATION OF TGF SIGNALING AND HOMEOBOX GENES

Increasing evidence indicates that cross-talk between members of the TGF super-family and of the *DLX* homeobox gene family is important for controlling normal bone morphogenesis and skeletal patterning (1, 180, 181). On one hand, signaling by several TGF super-family members controls transcriptional activity and/or expression of DLX transcription factors. For instance, BMP-2 activates *DLX3* transcription (182). In contrast, Smad6, an antagonist of BMP signaling, inhibits transcriptional activity of DLX3 by inhibiting its ability to bind target gene promoters (183). On the other hand, DLX proteins can control TGF signaling by modulating Smad activities. DLX1 has been reported to inhibit activin signaling by binding Smad4 (184). Cross-regulation between members of the TGF super-family and other homeobox genes has also been reported. For example, *Mixer* and *Milk*, members of the homeobox *Mix* family, enhance Smad-mediated transcription of *Goosecoid* in *xenopus* by interacting and recruiting activated Smad complexes to Mixer/Milk binding sites on the *Goosecoid* promoter (181). Group 13 HOX proteins interact with Smad1, Smad2 and Smad5 and block their transcriptional activities (185). Conversely, Smad1 has been reported to block transcriptional activity of HOXB4, HOXB7, HOXC8 and HOXD10 (186).

D. HYPOTHESIS AND SPECIFIC AIMS

The resistance to anti-growth signals is a major hallmark of cancer. TGF-β is a key cytokine that inhibits growth of most normal cells by inducing G_1 arrest (Figure 6). The anti-proliferative effect of TGF-β is orchestrated by a cytostatic program of gene responses that are controlled by Smad-dependent mechanisms (Figure 1). Central to this cytostatic program are activation of the CDK inhibitors, $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, and repression of the growth-promoting transcription factors, c-myc and Ids (Figure 6). Binding affinity and selectivity of Smad complexes for target gene promoters are governed by Smad interactions with other DNA-binding factors. Cooperative interactions between Smad proteins and the Sp1 transcription factor are central to TGF-β-mediated induction of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ transcription (Figure 4A). Most types of cancers are resistant to the anti-proliferative effect of TGF-β. This resistance has been attributed to TGF-β receptor and/or Smad mutations in some types of cancer (Table 2). However, the rareness of these mutations in many other tumors indicates that resistance to the anti-proliferative effect of TGF-β also stems from other molecular aberrations (Table 2).

DLX4 is a member of the *DLX* homeobox gene family. Unlike most other homeobox genes that have been studied to date, DLX4 is expressed in a wide range of different malignancies. Mounting evidence indicates that cross-talk between the members of the TGF super-family and *DLX* genes is important for controlling normal bone morphogenesis and skeletal patterning (Section C). My central hypothesis is that *DLX4* promotes tumor growth by modulating the TGF-β signaling pathway in tumors.

My overall goal is to determine whether and how DLX4 blocks the anti-proliferative effect of TGF-β in tumors.

The specific aims of my thesis project are to determine:

1) whether DLX4 inhibits TGF-β-induced, Smad-dependent responses

2) whether DLX4 blocks Smad transcription activity

3) whether DLX4 represses Smad/Sp1-mediated transcription

CHAPTER 2: MATERIALS AND METHODS

1. Plasmids

A *BP1* cDNA that contains the full-length coding region of human *DLX4* (172) was provided by Dr. Patricia Berg (George Washington University). FLAG-tagged *DLX4* was cloned into the pIRES-EGFP2 and pRetroQ vectors (Clontech, Palo Alto, CA). DNA fragments encoding different DLX4 domains were subcloned into pET41 GST vectors (Novagen, Gibbstown, NJ) as described in Figure 29A. *DLX4* shRNAs and non-targeting shRNAs were purchased from OriGene Technology (Rockville, MD). GST-tagged Smad2 and Smad4 plasmids were provided by Dr. Fang Liu (Rutgers University). *Smad2, Smad3* and *Smad4* cDNAs were purchased from OriGene Technology. MH2 and linker domains of Smad2 and Smad3, described in Figure 23, were subcloned into the pFA-CMV plasmid containing the GAL4-DBD (Stratagene, La Jolla, CA). The GAL4-driven pRF-Luc reporter construct was also purchased from Stratagene. The pGL2 F-Luc reporter vector was purchased from Promega. The *c-myc* promoter reporter construct, pBV-MYC(Del4) and pSBE4-Luc reporter construct containing four tandem SBE elements (193, 194) were provided by Dr. Bert Vogelstein (Johns Hopkins University). The Cignal Sp1 reporter construct containing a synthetic promoter comprising tandem Sp1-binding sites was purchased from SABiosciences (Frederick, MD). *Sp1* cDNA was provided by Dr. Keping Xie (MD Anderson Cancer Center). pGL3 reporter constructs (p15-WT and p15-SBE-mt) containing wild-type and mutant $p15^{Ink4B}$ promoter sequences (51) were provided by Drs. Xiao-Fan Wang (Duke University Medical Center) and Xin-Hua Feng (Baylor College of Medicine). The *Id1*

promoter construct was provided by Dr. Robert Benezra (Memorial Sloan-Kettering Cancer Center) (195). The BRE-Luc reporter construct (196) was provided by Dr. Peter ten Dijke (Netherlands Cancer Institute).

2. Antibodies and other reagents

Antibodies (Abs) were purchased from commercial sources as follows: DLX4 Abs for immunoblotting Abcam (Cambridge, MA) and Abnova Corporation (Taipei, Taiwan), for immunoprecipitation (Santa Cruz Biotechnology, Santa cruz, CA). Smad2, phospho-Smad2 (Ser465/467), Smad3, Smad4, $p15^{Ink4B}$, c-myc (Cell Signaling Technology, Danvers, MA); $p21^{WAF1/Cip1}$ (Calbiochem, Gibbstown, NJ); Sp1, E-cadherin (Zymed Laboratories, Carlsbad, CA); N-cadherin (BD Biosciences, San Jose, CA); actin, FLAG-M2 (Sigma-Aldrich, St. Louis, MO); lamin A/C and Smad2/3 (Santa Cruz Biotechnology), HRP-conjugated secondary Abs (Bio-Rad, Hercules, CA), Alexa Fluor 594-conjugated secondary Abs (Invitrogen, Carlsbad, CA). Recombinant Sp1 protein, TGF-β and BMP-4 were purchased from Promega (Madison, WI), Sigma-Aldrich and R&D Systems (Minneapolis, MN), respectively.

3. Cell lines

HepG2 cells were provided by Dr. Michelle Barton (MD Anderson Cancer Center). Mv1Lu cells were purchased from American Type Culture Collection (Manassas, VA). Both cell lines were cultured in MEM medium supplemented with 10% FBS, 2mM glutamine and penicillin-streptomycin. NMuMG and MDA-MB-468 cells were purchased from American Type Culture Collection (Manassas, VA) and

cultured in GIBCO® RPMI-1640 Medium supplemented with 10% FBS, 2mM glutamine and penicillin-streptomycin. Ampho-293 cells (provided by Dr. Douglas Boyd, MD Anderson Cancer Center) and MCF-7 cells (provided by Dr. Francois-Xavier Claret, MD Anderson Cancer Center) were cultured in DMEM medium supplemented with 10% FBS, 2mM glutamine and penicillin-streptomycin.

4. Protein over-expression and knock-down

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 days thereafter and used to infect target cells. Stable lines were selected by puromycin (0.5 μg/ml). For studying Smad-dependent growth inhibition, Smad4 was transiently expressed in MDA-MB-468 cells that lack Smad4. For transient expression, cells were transfected with recombinant plasmids using FuGENE6 reagent following manufacturer's instructions (Roche Applied Biosciences, Indianapolis, IN). To assay the effect of DLX4 knock-down, MCF-7 cells were transfected with shRNA constructs (empty vector, non-targeting and two DLX4 shRNAs) purchased from OriGene Technology using FuGENE6 reagent.

5. Cell growth assays

Cells were seeded in 96-well plates at 4,000 cells per well in 100 μl medium and cultured for 2 days in complete medium containing 0, 1, 3, 10, 30 and 100 ng/ml TGF-β. MTT assays were performed following manufacturer's instructions (Roche

Applied Biosciences). Briefly, 10 μl of MTT labeling reagent was added to each well (final MTT concentration 0.5 mg/ml). The plate was incubated for 4 hours in a humidified atmosphere (37°C, 5% CO_2) then 100 μl of solubilization solution was added to each well. The plate was incubated overnight in a humidified atmosphere. The spectrophotometrical absorbance (570 nm) was measured. Experiments were done in triplicate and repeated two times.

6. 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 (10 ng/ml) for 18 h. Cells were harvested and washed in phosphate-buffered saline (PBS). Cells were then fixed in 1 ml of 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes on ice. Cells were washed in PBS, resuspended in 1 ml of cold 70% ethanol and kept at -20^0C. Following centrifugation at 800 x g, cells were washed with PBS then stained with 1 ml of staining solution containing 40 μg/ml propidium iodide (Sigma-Aldrich) and RNaseA at 37^0C for 30 minutes in the dark. Distribution throughout the cell cycle was determined by flow cytometric analysis.

7. Reporter Assays

Cells were seeded at 4–5 x 10^4 cells/well in 12-well plates and co-transfected with expression plasmids (400 ng), reporter plasmid (100 ng) and pRL-CMV Renilla luciferase (R-Luc) reporter plasmid (0.5 ng) (Promega) for normalizing transfection

efficiency using FuGENE6 reagent (Roche Applied Biosciences). At 24 hours after transfection, cells were cultured for an additional 18 hours with and without TGF-β or BMP-4. Luciferase activities were assayed using the Dual-reporter assay kit (Promega) and measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Experiments were performed in triplicate and repeated two times. GAL4 reporter assay were performed as above with some modifications. Briefly, DLX4 or empty vector was co-transfected with GAL4-driven reporter construct and GAL4-DBD/Smad2 and GAL4-DBD/Smad3. TGF-β treatment and luciferase assays were performed as described above.

8. Immunofluorescence staining

Cells ($5x10^4$/well) were seeded in 2-well chamber slides. Cells were serum starved overnight and then treated with TGF-β for 1 hour. Cells were washed with PBS and fixed with 1% paraformaldehyde (in PBS, pH 7.4) for 20 minutes on ice. Fixed cells were washed with PBS two times and permeablized with 0.1% Triton X-100 (in PBS) for 20 minutes on ice. Cells were then washed with PBS and blocked with 1% goat serum in PBS for 30 minutes at 4^0C and stained with Abs to Smad2, DLX4, E-cadherin, or FLAG Ab (1:200). Cells were washed 5 times in PBS containing 1% BSA. Staining was detected by Alexa Fluor 594-conjugated secondary Ab (1:1000). Cells were also stained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) to visualize nuclei.

9. Immunoblotting

To determine protein expression levels, cells were lysed in M-PER buffer (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Total cell lysates were electrophoresed on 6 - 15% SDS gels, then transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ) and blocked with 5% nonfat milk in PBS with 0.1% Tween-20 (PBS-T). Immunoblotting was performed with primary Abs at recommended dilution provided by manufacturers in PBS-T containing 5% nonfat milk except for phospho Smad2 Ab which was prepared in PBS-T with 5% BSA. Membranes were hybridized with primary Abs overnight at 4^{o}C then washed with PBS-T buffer. Secondary Abs were diluted at 1:2000 in PBS-T with 5% nonfat milk and incubated with membranes for 1-2 hours at room temperature. Membranes were then washed with PBS-T buffer and incubated with ECL Western blotting detection reagent (Amersham Biosciences) and exposed to autoradiographic film.

10. Immunoprecipitation

Cell lysate preparation:

Cells were plated to reach 70-80% confluence and were transfected with DLX4 or empty vector. At 24 hours after transfection, cells were serum-starved overnight and then treated without or with 10 ng/ml TGF-β for 1 hour. To prepare whole cell lysates, cells were washed with PBS buffer and lysed in native buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA) supplemented with protease and phosphatase inhibitors for 10 minutes on ice. Cells were then sonicated. To

reduce viscosity caused by genomic DNA, benzonase enzyme was added. Lysates were centrifuged at 12000 x g for 10 minutes and supernatants were collected. Whole cell lysates were used immediately for immunoprecipitation or stored at -80^{o}C. To prepare nuclear extracts, cells were lysed in cytosol 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 x g for 10 minutes. Nuclear pellets were washed then lysed in native buffer (Tris pH 8.0 20mM, 100mM NaCl, NP40 1%, Glycerol 10% EDTA 2mM) plus protease and phosphatase inhibitors. The lysates were sonicated then centrifuged at 12000 x g for 10 minutes. Supernatants were transferred to a fresh eppendorf tube. Nuclear lysates were used immediately for immunoprecipitation or stored at -80^{o}C

Immunoprecipitation:

Whole cell extracts (1 mg) or nuclear extracts (500 ug) were pre-cleared with protein G agarose (Amersham Biosciences) by rotating at 4°C for 30 minutes. Lysates were then incubated with anti-FLAG-M2 affinity gel (Sigma-Aldrich) for 4-12 hours at 4°C. Where unconjugated primary Abs were used, lysates were incubated with the Abs for 12 hours at 4°C then further incubated with protein G agarose for 1 hour at 4°C. Immunoprecipitates were washed five times with native buffer and subjected to SDS PAGE and immunoblot analysis.

11. Oligonucleotide pull-down assays

Preparation of biotinylated oligonucleotides:

Sense and antisense oligomers were designed to cover the p15^{Ink4B} promotor region from positions -108 to -39 (Figure 31A and Table 6). The sense oligomer was labeled with biotin at the 5' end. Oligos were generated by Sigma-Aldrich. Oligomers were dissolved in H_2O. Oligomers were annealed by incubating at 98^{o}C for 10 minutes followed by slow cooling down to room temperature overnight. The annealed product was verified by agarose DNA electrophoresis.

DNA pull-down:

Nuclear lysates were prepared as described for immunoprecipitation except that lysates were dissolved in 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) plus proteinase inhibitor cocktail. Nuclear lysates were pre-cleared with strepavidin agarose for 30 minutes at 4°C. Biotin-labeled oligonucleotides were added to the lysate and incubated overnight at 4°C with rotating. The lysate was then added to pre-washed streptavidin-conjugated agarose beads and further rotated at 4°C for 1 hour. Beads were washed 4 times with binding buffer. DNA bound proteins were eluted from strepavidin-conjugated agarose beads with 1X SDS sample (Laemmli) buffer at 85°C for 5 minutes. The samples were then subjected to SDS-PAGE and immunoblotting.

Table 6. p15*Ink4B* oligomers for oligonucleotide pull-down assays

Name	Sequence
Sense strand	5'-BIOTIN-GCCTGGCCTCCCGGCGATCACAGCGGACAGGGGGCGGAGCCTAAGGGGGTGGGGAGACGCCGGCCCCTTG-3’
Antisense strand	5'-AAGGGGCCGGCGTCTCCCCACCCCCTTAGGCTCCGCCCCCTGTCCGCTGTGATCGCCGGGAGGCCAGGC-3'

12. *In vitro* binding assays

Preparation of GST fusion and ^{35}S-labeled proteins:

GST fusion proteins containing full-length DLX4, truncated DLX4, Smad2 and Smad4 were produced in *E.coli* and purified by glutathione beads (Amersham Biosciences) following the manufacturer's instructions. ^{35}S-labeled DLX4, Smad4 and Sp1 were synthesized *in vitro* from the *T7* promoter using the TNT coupled translation kit (Promega) following the manufacturer's instructions.

Binding assays:

GST fusion protein (1 μg) bound to glutathione-sepharose beads was pre-incubated with 0.5 mg/ml BSA in binding buffer (20 mM Tris [pH8.0], 100 mM NaCl, 0.1% NP40, 2 mM EDTA, plus proteinase inhibitor cocktail) for 1 hour. ^{35}S-labeled proteins were pre-cleared by incubating with glutathione-sepharose beads in binding buffer for 1 hour. GST fusion protein was then incubated with pre-cleared ^{35}S-labeled protein for 2 hours. Beads were then extensively washed with binding buffer for 5 times. Associated proteins were subjected to SDS-PAGE and visualized by autoradiography.

13. Total RNA extraction and qPCR

Total RNA extraction:

Total RNA was extracted using TRIzol reagent (Invitrogen) following manufacturer's instructions. Extracted RNA was treated with DNAseI and purified using the RNeasy kit (Qiagen, Valencia, CA).

qPCR:

Purified total RNA (500 ng) was used to synthesize cDNA by random priming using the RT² First Strand Kit (SABiosciences). cDNA and primers were included in a reaction (20 ul) with the RT² SYBR® Green qPCR Master Mix (SABiosciences). Reactions were run on the AB7500 system (Applied Biosystems, Carlsbad, CA) with 40 cycles of 95^0C for 15 seconds and 60^0C for 1 minute according to the standard manufacturer's program. Primers were predesigned to amplify a 98 bp region located at positions 1278-1297 of the 3' untranslated region of *DLX4* gene (SABioscience). Primer specificity was confirmed by including a dissociation curve at the end of thermal cycles. Data analysis was performed using the delta-delta Ct method. Levels of *DLX4* expression were normalized to β-actin.

14. Gel-shift assays

^{32}P-labeled probe preparation:

Oligomers were designed to contain sense and antisense sequences -88 to -64 of the p15^{Ink4B} promoter with 5' overhangs for labeling (Figure 35A and Table 7). Double-stranded oligos were generated by incubating sense and antisense oligomers at 98^0C for 10 minutes followed by slow cooling to room temperature overnight. ^{32}P-labeled probe was produced by end-filling using the Klenow enzyme (Roche Applied Biosciences) following manufacturer's instructions. The ^{32}P-labeled probe was then purified through NICK™ Column (Amersham Biosciences) and eluted in TE buffer to a final concentration of 1 ng/ul.

Table 7. p15*lnk4B* oligomers for gel-shift assays

Name	Sequence
Sense strand	5'-CAGCGGACAGGGGGCGGAGCCTAAG-3′
Antisense strand	5'-CTTAGGCTCCGCCCCCTGT-3'

Gel-shift reaction:

EMSA was performed in 25 μl reactions. Recombinant Sp1 protein (100 ng) was incubated with increasing amounts of *in vitro* translated FLAG-DLX4 and FLAG-tag synthesized from TNT coupled reticulocyte lysates (Promega) for 20 minutes 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(dI-dC).poly(dI-dC)) at 23^0C. 1ng of ^{32}P-labeled probe was added to the binding reaction and incubated for an additional 20 minutes. The reactions were electrophoresed on a 5% nondenaturing polyacrylamide gel. The gel was dried and exposed to autographic film.

15. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the ChIP Assay Kit (Upstate; Temecula, CA) following manufacturer's instructions with modifications. Briefly, cells were cross-linked by adding formaldehyde to 1% final concentration at room temperature for 10 minutes. Formaldehyde was then quenched by adding glycine and incubated at room temperature for 5 minutes. The cells were then washed with PBS and scraped off dishes. Cell suspensions were centrifuged and the supernatant was discarded. Cells were lysed in SDS lysis buffer containing protease inhibitors and sonicated to generate DNA fragments of ~200-1000 base pairs in length. Sheared chromatin was then pre-cleared with Protein G agarose then incubated with 4 ug of Abs to Sp1 (Zymed Laboratories), Smad4 (Cell signal), Smad2/3, and normal IgG (Santa Cruz Biotechnology) overnight. Protein G agarose was then added and incubated for 1 hour. The protein G agarose-antibody/chromatin complex was

extensively washed, followed by elution of immunoprecipitated complexes and reversal of cross-links. Protein and RNA were removed by incubating with RNase A and proteinase K. Eluted DNA fragments were purified and used for PCR reactions to amplify a 535 bp fragment of the p15^{Ink4B} promoter (Table 8). The amplified fragment was confirmed by DNA sequencing.

Table 8. p15*Ink4B* primer sequences for ChIP PCR

Name	Sequence
Sense strand	5'-TATGGTTGACTAATTCAAACAG-3′
Antisense strand	5'-GCAAAGAATTCCGTTTTCAGCT-3'

CHAPTER 3: DLX4 INHIBITS TGF-β-INDUCED, SMAD-DEPENDENT RESPONSES

A. RATIONALE

TGF-β inhibits growth of most types of normal cells by inducing cell cycle arrest. The anti-proliferative effect of TGF-β is orchestrated by a cytostatic program of gene responses that principally involves activation of the CDK inhibitors $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ (38, 51) and repression of the growth-promoting transcription factors c-myc and Ids (52, 59) (Figure 6). In many types of tumors, the anti-proliferative effect of TGF-β is abolished (3, 4). Resistance to the anti-proliferative effect of TGF-β has been attributed to TGF-β receptor and/or Smad mutations in some types of tumors, particularly those of gastrointestinal origin (112, 114, 115) (Table 2). However, the rareness of these mutations in many other types of tumors indicates that resistance to the anti-proliferative effect of TGF-β also stems from other aberrations (112) (Table 2).

Cross-talk between members of the *DLX* gene family and the TGF super-family is important for controlling bone morphogenesis and skeletal patterning (1, 180, 181). The homeobox gene *DLX4* is not expressed in most normal adult tissues, but is expressed in a wide range of tumors (Table 5). This raises the possibility that *DLX4* promotes tumorigenesis via a mechanism common to multiple types of tumors. The goal of my studies in this chapter is to determine whether DLX4 blocks TGF-β-induced, Smad-dependent responses that are central to the anti-proliferative effect of TGF-β.

B. RESULTS

1. DLX4 blocks TGF-β-mediated growth inhibition

To determine whether DLX4 blocks the anti-proliferative effect of TGF-β, we first assayed the effect of DLX4 on cell growth in the non-tumorigenic lung epithelial cell line Mv1Lu. Mv1Lu is a well-established model for studying TGF-β-induced growth arrest (197, 198). Growth of Mv1Lu cells was inhibited by TGF-β in a dose-dependent manner (Figure 14A). In contrast, enforced expression of DLX4 in Mv1Lu cells decreased the sensitivity to TGF-β (Figure 14A). This observation indicates that DLX4 blocks the growth-inhibitory effect of TGF-β. To confirm this finding, the effect of knocking-down DLX4 on cell growth was assayed. DLX4 was knocked-down by using shRNAs that targeted different sites of *DLX4* (sh90 and sh92). Knockdown of DLX4 in MCF-7 breast cancer cells increased sensitivity to TGF-β in cell viability assays (Figure 14B). The ability of these shRNAs to knock-down DLX4 in MCF-7 cells was confirmed by Western blot (Figure 15A) and also by qPCR and immunofluorescence staining (Figure 15B, 15C).

Figure 14. DLX4 blocks TGF-β-mediated growth inhibition

[A] Vector-control (-DLX4) and +DLX4 stable Mv1Lu lines were cultured with the indicated concentrations of TGF-β for 2 days. 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]** Transfected MCF-7 cells were cultured with the indicated concentrations of TGF-β for 2 days. Changes in cell growth were determined by MTT assay.

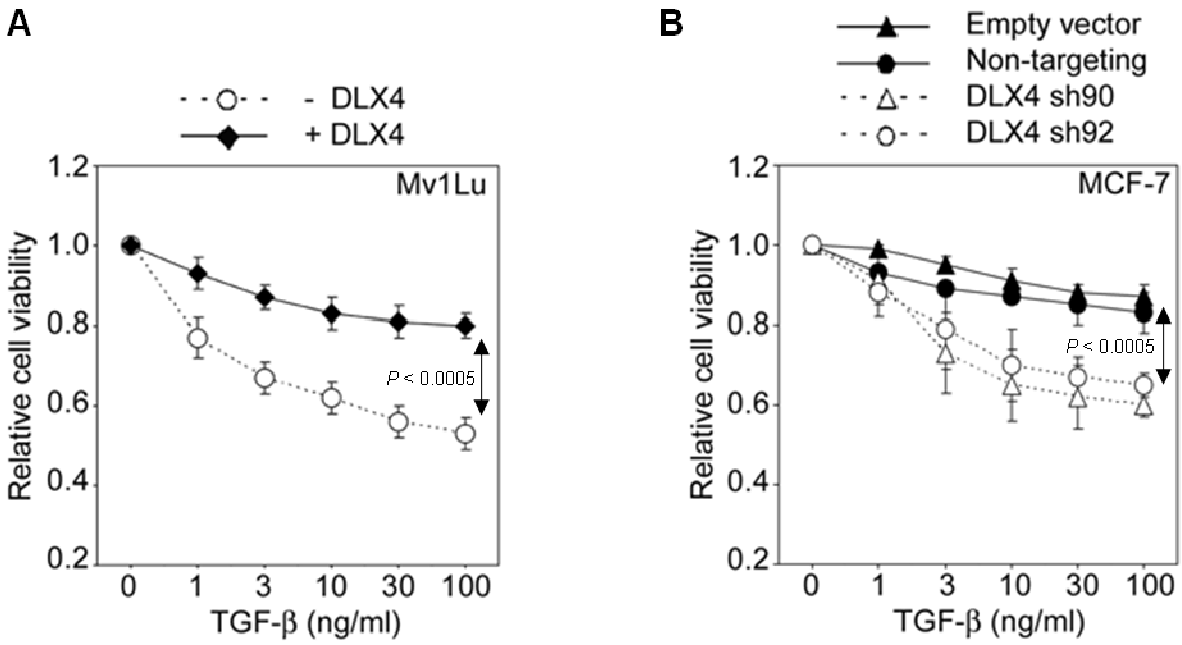
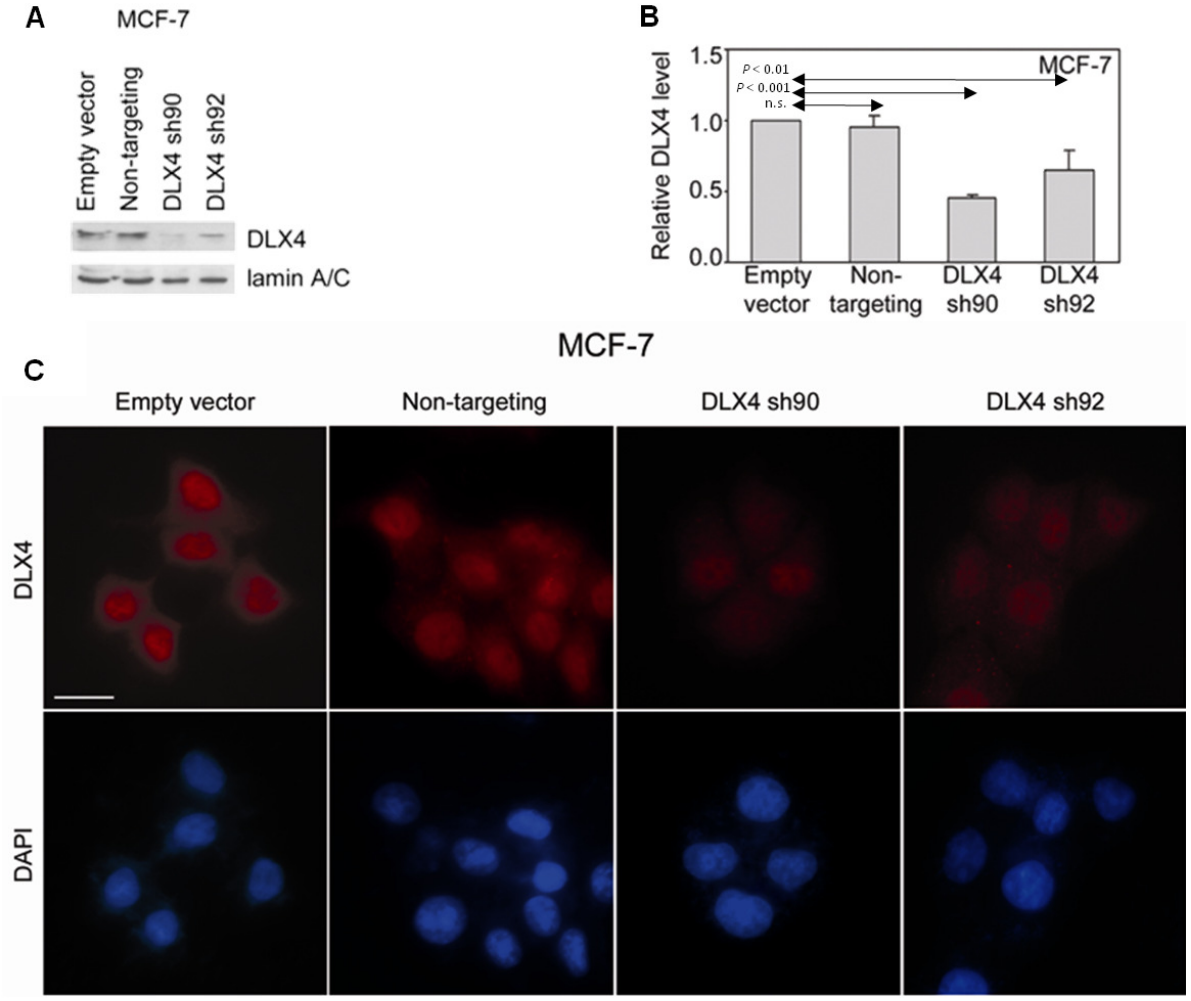


Figure 15. Knockdown of DLX4

MCF-7 cells were transfected with empty vector, non-targeting shRNA and *DLX4* shRNAs (sh90, sh92). **[A]** At 2 days after transfection, DLX4 levels were assayed by Western blot. **[B]** *DLX4* transcript levels in transfected MCF-7 cells were assayed by qPCR using SYBR®Green qPCR Master Mix and *DLX4* primers purchased from SABiosciences. **[C]** At 2 days after transfection, endogenous DLX4 in cells was detected by staining using DLX4 Ab (red). Nuclei were visualized by staining with DAPI (blue). Bar, 20 μm.



2. DLX4 blocks the ability of TGF-β to induce G_1 arrest

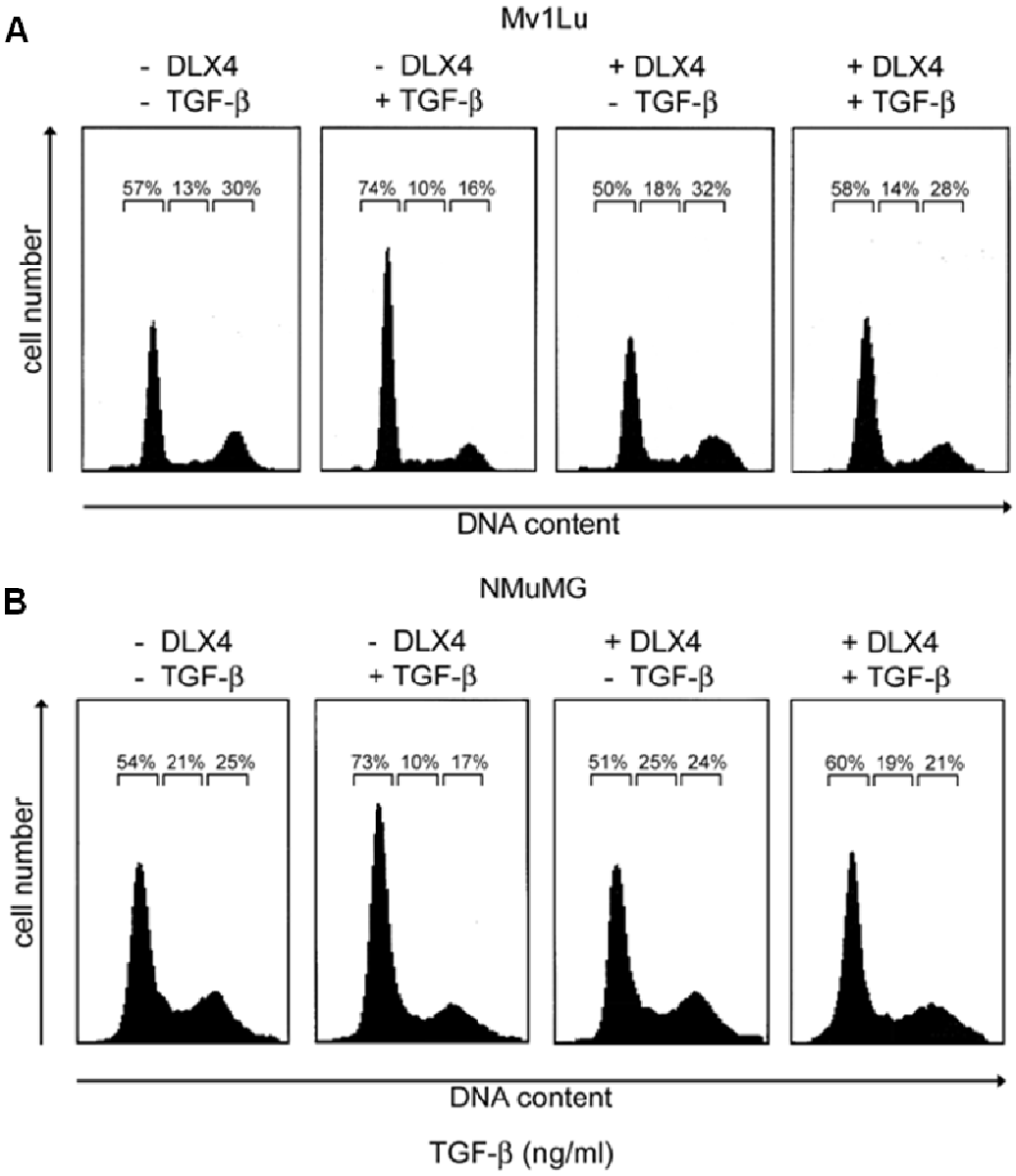
TGF-β induces cell cycle arrest in G_1 phase (198, 199). We determined whether DLX4 blocks the ability of TGF-β to induce G_1 arrest by performing cell cycle analysis. Treatment of vector-control Mv1Lu cells with TGF-β led to significant accumulation of cells in G_1 phase (Figure 16A). However, enforced expression of DLX4 in Mv1Lu cells inhibited the induction of G_1 arrest by TGF-β (Figure 16A). Similarly, enforced expression of DLX4 in the non-tumorigenic mammary epithelial cell line NMuMG also inhibited TGF-β-induced G_1 arrest (Figure 16B). Conversely, knockdown of DLX4 in MCF-7 cells was observed to increase the proportion of cells in G_1 phase (Figure 16C). These data indicate that DLX4 inhibits TGF-β-mediated G_1 arrest.

3. DLX4 blocks Smad-dependent growth inhibition

TGF-β inhibits cell growth principally via Smad-dependent mechanisms that require Smad4 (3). Because TGF-β can also inhibit cell growth via Smad-independent mechanisms (60), we determined whether DLX4 blocks Smad-dependent growth inhibition. The effect of DLX4 on cell growth was assayed using the MDA-MB-468 breast cancer cell line that has the homozygous deletion of *Smad4* (122). Growth of MDA-MB-468 cells was not inhibited by TGF-β (Figure 17). In contrast, reconstitution of Smad4 in these cells increased responsiveness to TGF-β (Figure 17). This Smad4-dependent responsiveness to TGF-β was abrogated when DLX4 was expressed (Figure 17). These results confirm our finding that DLX4 opposes the anti-proliferative

Figure 16. DLX4 blocks TGF-β-induced G_1 arrest

[A] Mv1Lu lines were treated without and with 10 ng/ml TGF-β for 18 hours. **[B]** Transfected MCF-7 cells were treated without and with TGF-β (10 ng/ml) for 18 hours. **[C]** Vector-control (-DLX4) and +DLX4 NMuMG cells were treated without and with TGF-β (5 ng/ml) for 18 hours. Indicated are the proportions of cells in G_1, S and G_2/M phases determined by flow cytometric analysis of propidium iodide-staining.


A
Mv1Lu
- DLX4
- TGF-β
- DLX4
+ TGF-β
+ DLX4
- TGF-β
+ DLX4
+ TGF-β
57% 13% 30%
74% 10% 16%
50% 18% 32%
58% 14% 28%
cell number
DNA content
B
NMuMG
- DLX4
- TGF-β
- DLX4
+ TGF-β
+ DLX4
- TGF-β
+ DLX4
+ TGF-β
54% 21% 25%
73% 10% 17%
51% 25% 24%
60% 19% 21%
cell number
DNA content
TGF-β (ng/ml)

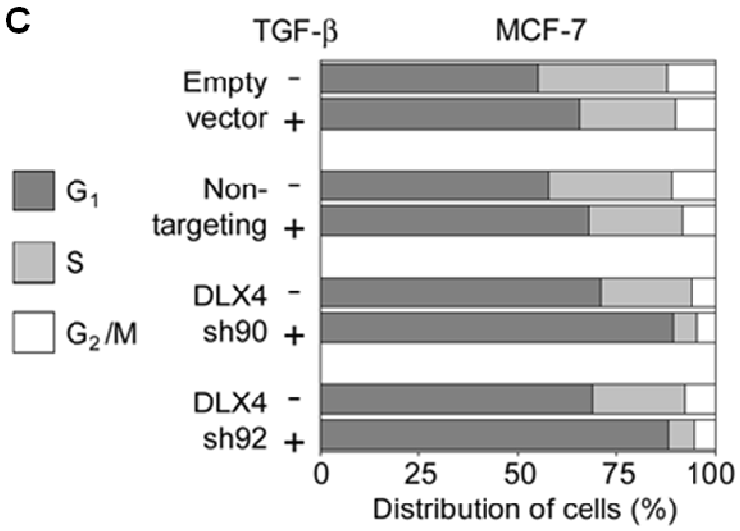
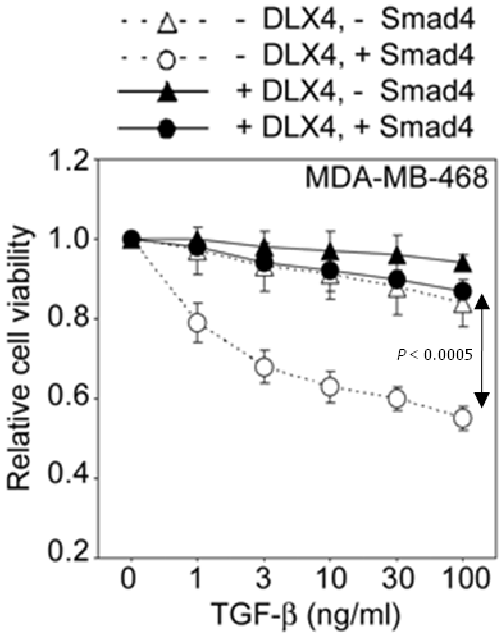

C
TGF-β
MCF-7
Empty vector
Non-targeting
DLX4 sh90
DLX4 sh92
-
+
G1
S
G2/M
0
25
50
75
100
Distribution of cells (%)

Figure 17. DLX4 blocks Smad-dependent growth inhibition

Vector-control (-DLX4) and +DLX4 stable MDA-MB-468 lines were transfected with Smad4. At 24 hours thereafter, cells were cultured without and with TGF-β (10 ng/ml) for 2 days and changes in cell growth were examined by MTT assay.



effect of TGF-β, and indicate that DLX4 blocks TGF-β-mediated growth inhibition in a Smad-dependent manner.

4. DLX4 inhibits gene responses of the TGF-β cytostatic program

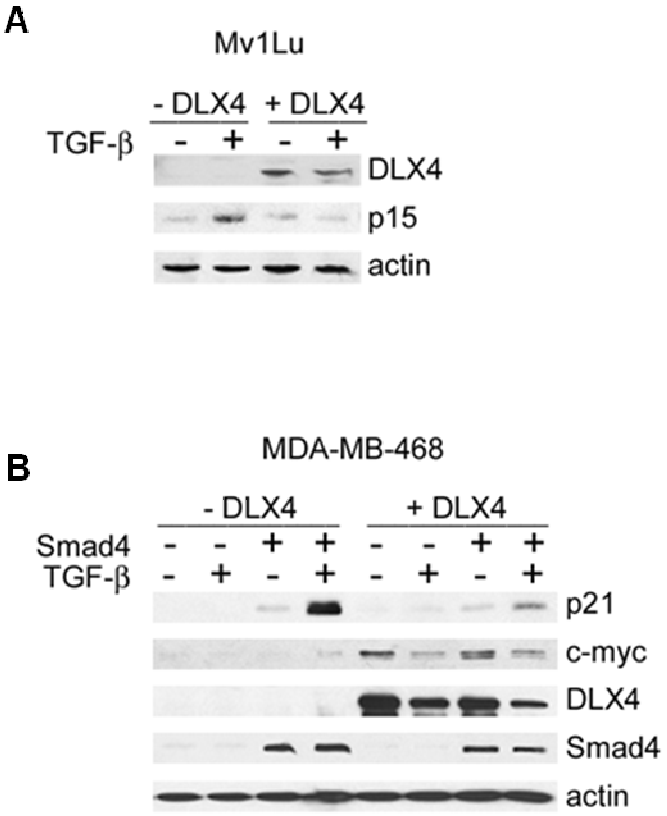
TGF-β inhibits cell growth by controlling a cytostatic program of gene responses that includes activation of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ transcription and repression of c-*myc* and *Id* transcription (38, 51, 52, 59). In subsequent experiments, we determined whether DLX4 inhibits gene responses of this cytostatic program.

4.1. DLX4 inhibits TGF-β-mediated induction of CDK inhibitors

Treatment of vector-control Mv1Lu cells with TGF-β induced expression of $p15^{Ink4B}$ (Figure 18A). However, enforced expression of DLX4 in Mv1Lu cells blocked TGF-β-induced $p15^{Ink4B}$ expression (Figure 18A). To determine whether this blocking effect was Smad-dependent, we assayed the expression of TGF-β response genes in MDA-MB-468 cells. Treatment of MDA-MB-468 cells with TGF-β did not induce expression of $p21^{WAF1/Cip1}$ (Figure 18B). When MDA-MB-468 cells were reconstituted with Smad4, $p21^{WAF1/Cip1}$ expression was induced by TGF-β. However, enforced expression of DLX4 blocked this induction (Figure 18B). These results indicate that DLX4 inhibits TGF-β-mediated, Smad-dependent induction of CDK inhibitor expression.

Figure 18. DLX4 blocks Smad-dependent cytostatic gene responses

[A] Western blot analysis of Mv1Lu lines following treatment without and with TGF-β (10 ng/ml) for 16 hours. **[B]** Western blot analysis of MDA-MB-468 lines following treatment without and with TGF-β for 16 hours.

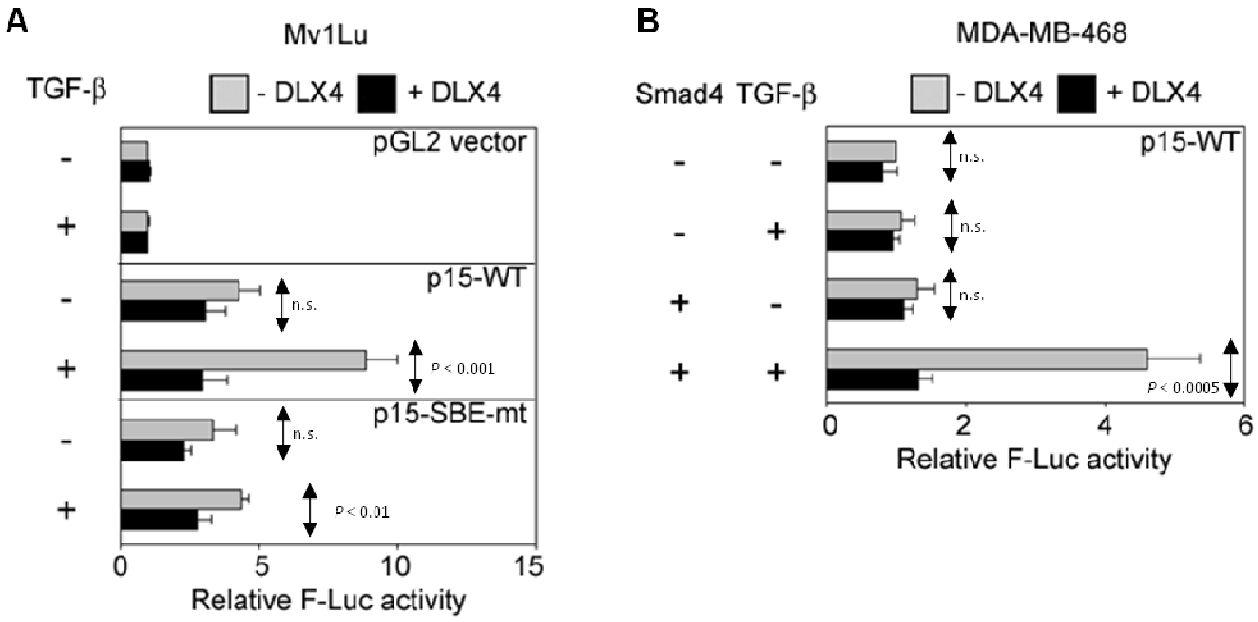


4.2. DLX4 blocks Smad-dependent transcription of p15^{Ink4B}

TGF-β induces transcription of p15^{Ink4B} and p21$^{WAF1/Cip1}$ by similar Smad-dependent mechanisms (38, 51). Because DLX4 is a transcription factor, it is likely that DLX4 blocks Smad-mediated transcription of these genes. To investigate this possibility, we focused on the well-characterized p15^{Ink4B} promoter. The first 113 bp of the p15^{Ink4B} promoter are essential for induction by TGF-β and contain two Smad-binding elements (SBEs) (51). We initially determined whether DLX4 inhibits the ability of TGF-β to induce p15^{Ink4B} promoter activity by reporter assays using a construct driven by the minimal p15^{Ink4B} promoter (-113 to +70) (p15-WT). Activity of this minimal promoter was induced by TGF-β in vector-control Mv1Lu cells, and this induction was abolished by mutation of the SBEs (Figure 19A). Enforced expression of DLX4 in Mv1Lu cells abolished the induction of wild-type p15^{Ink4B} promoter activity by TGF-β (Figure 19A). DLX4 also modestly inhibited activity of the SBE-mutant promoter (Figure 19A). This result suggests that DLX4 also can block basal p15^{Ink4B} promoter activity independently of TGF-β/Smad signaling. To confirm that DLX4 blocks Smad-mediated induction of p15^{Ink4B}, we assayed p15^{Ink4B} promoter activity in Smad4-deficient MDA-MB-468 cells. Wild-type p15^{Ink4B} promoter activity was unresponsive to TGF-β in MDA-MB-468 cells. TGF-β responsiveness was conferred when Smad4 was expressed in these cells. However, this Smad4-dependent responsiveness was eliminated when DLX4 was co-expressed (Figure 19B). Together, these findings demonstrate that DLX4 blocks Smad-mediated transcription of p15^{Ink4B}.

Figure 19. DLX4 inhibits TGF-β-mediated induction of p15^{Ink4B} promoter activity

[A] 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 hours, and assayed for F-Luc activity. **[B]** 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.

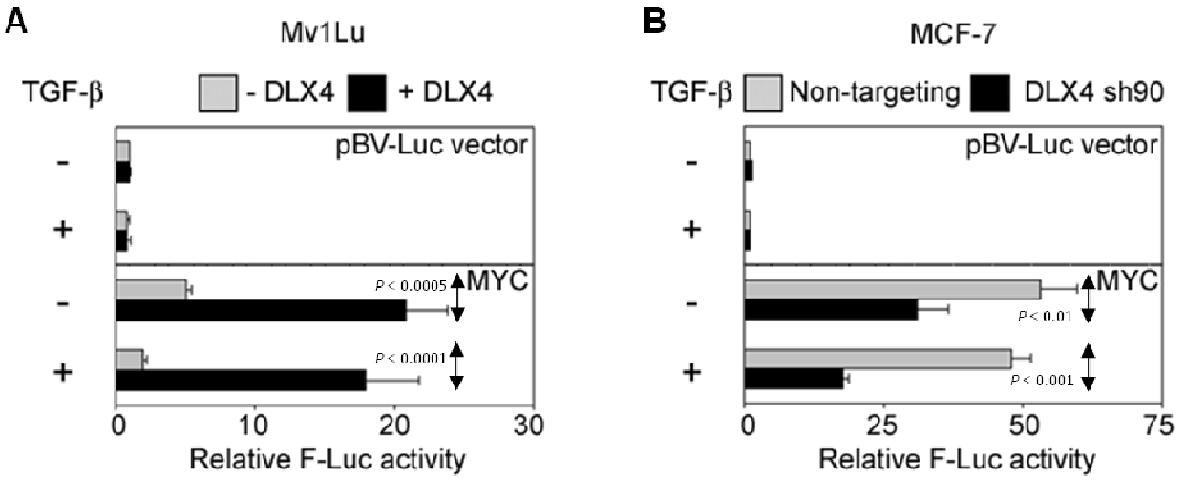


4.3. DLX4 induces c-*myc* expression independently of TGF-β/Smad signaling

In subsequent experiments, we determined whether DLX4 blocks TGF-β-mediated repression of c-myc expression. Enforced expression of DLX4 induced c-myc expression in MDA-MB-468 cells, irrespective of the absence or presence of Smad4 (Figure 18B). Because DLX4 induced the level of c-myc protein independently of TGF-β/Smad signaling, we 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 vector-control Mv1Lu cells (Figure 20A). However, enforced expression of DLX4 in Mv1Lu cells induced c-*myc* promoter activity both in the absence and presence of TGF-β stimulation (Figure 20A). In converse experiments, knock-down of *DLX4* inhibited c-*myc* promoter activity in MCF-7 cells in the absence of TGF-β stimulation (Figure 20B). TGF-β treatment of DLX4 knock-down cells further inhibited c-*myc* promoter activity (Figure 20B). These results suggest that DLX4 blocks the ability of TGF-β to repress c-myc expression and can also induce c-myc expression independently of TGF-β/Smad signaling.

Figure 20. DLX4 induces c-*myc* 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 hours, 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).

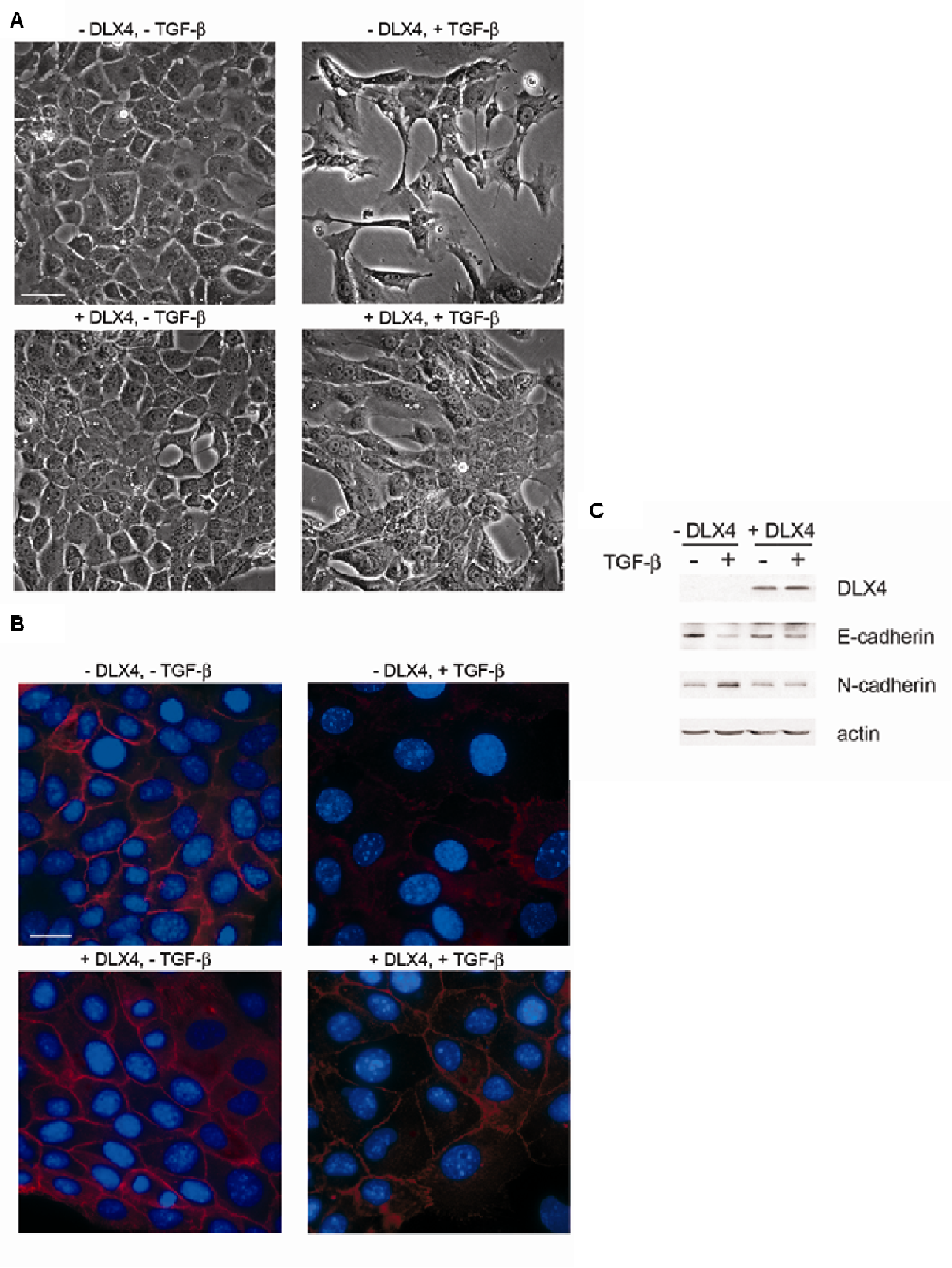


5. DLX4 inhibits TGF-β-induced EMT

In addition to its anti-proliferative effect, TGF-β is well-known to induce EMT via Smad and non-Smad mechanisms (3, 4) (Figure 8). Because our results demonstrate that DLX4 blocks TGF-β-mediated growth inhibition in a Smad-dependent manner, it is possible that DLX4 also blocks the ability of TGF-β to induce EMT. To address this possibility, 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 (200). TGF-β treatment of vector-control NMuMG cells induced profound epithelial-to-fibroblastic morphologic transformation (Figure 21A). E-cadherin expression was lost, whereas N-cadherin was induced in vector-control NMuMG cells following TGF-β treatment (Figures 21B, 21C). In contrast, enforced expression of DLX4 in NMuMG cells blocked the down-regulation of E-cadherin and induction of N-cadherin (Figures 21B, 21C). Additionally, epithelial morphology was considerably retained in TGF-β-treated +DLX4 NMuMG cells (Figure 21A). These results indicate that DLX4 can inhibit TGF-β-induced EMT.

Figure 21. Effect of DLX4 on TGF-β-induced EMT

Vector-control (-DLX4) and +DLX4 NMuMG cells were incubated without or with TGF-β (5 ng/ml) for 24 hours. **[A]** Morphology of cells was visualized by phase-contrast microscopy. Bar, 50 μm. **[B]** E-cadherin expression was detected by immunofluorescence staining (red). Nuclei were visualized by staining with DAPI (blue). Bar, 20 μm. **[C]** Western blot analysis of DLX4, E-cadherin and N-cadherin.


A
- DLX4, - TGF-β
- DLX4, + TGF-β
+ DLX4, - TGF-β
+ DLX4, + TGF-β
B
- DLX4, - TGF-β
- DLX4, + TGF-β
+ DLX4, - TGF-β
+ DLX4, + TGF-β
C
- DLX4
+ DLX4
TGF-β
- + - +
DLX4
E-cadherin
N-cadherin
actin

C. CONCLUSION

The studies in this chapter demonstrate that DLX4 blocks the anti-proliferative effect of TGF-β by inhibiting the TGF-β-mediated cytostatic program of gene responses that cause G_1 arrest. The studies demonstrate that DLX4 blocks the ability of TGF-β to induce $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ expression, and that this blocking effect occurs via Smad-dependent mechanisms. In addition, the studies demonstrate that DLX4 blocks the ability of TGF-β to repress c-*myc* expression, and can also induce c-*myc* expression independently of TGF-β/Smad signaling. Furthermore, our findings indicate that DLX4 blocks the ability of TGF-β to induce EMT in cells that normally undergo TGF-β-induced EMT in a Smad-dependent manner. Together, our findings that DLX4 blocks TGF-β-mediated, Smad-dependent growth inhibition and also EMT indicate that DLX4 inhibits a core component of the TGF-β/Smad signaling pathway. Identification and characterization of this inhibitory mechanism will be the focus of Chapter 4 and Chapter 5.

CHAPTER 4: DLX4 BLOCKS SMAD TRANSCRIPTIONAL ACTIVITY

A. RATIONALE

Binding of TGF-β to TβRII leads to recruitment and activation of TβRI which in turn phosphorylates R-Smads. Phosphorylated R-Smads translocate to the nucleus, where they form heteromeric complexes with Smad4 and other DNA-binding factors to regulate gene transcription (9, 12) (Figure 2). TGF-β/Smad signaling is modulated at multiple levels. These include binding of TGF-β to TβRII, formation of the TβRI-TβRII complex, phosphorylation and nuclear localization of R-Smads, and transcriptional activity of Smad proteins (Figure 2).

Studies in Chapter 3 demonstrated that DLX4 blocks TGF-β-mediated gene responses through Smad-dependent mechanisms. Because DLX4 is a transcription factor, we hypothesize that DLX4 blocks TGF-β signaling by interfering with Smad transcriptional activity. The goal of the studies in this chapter is to determine whether and how DLX4 blocks Smad activity.

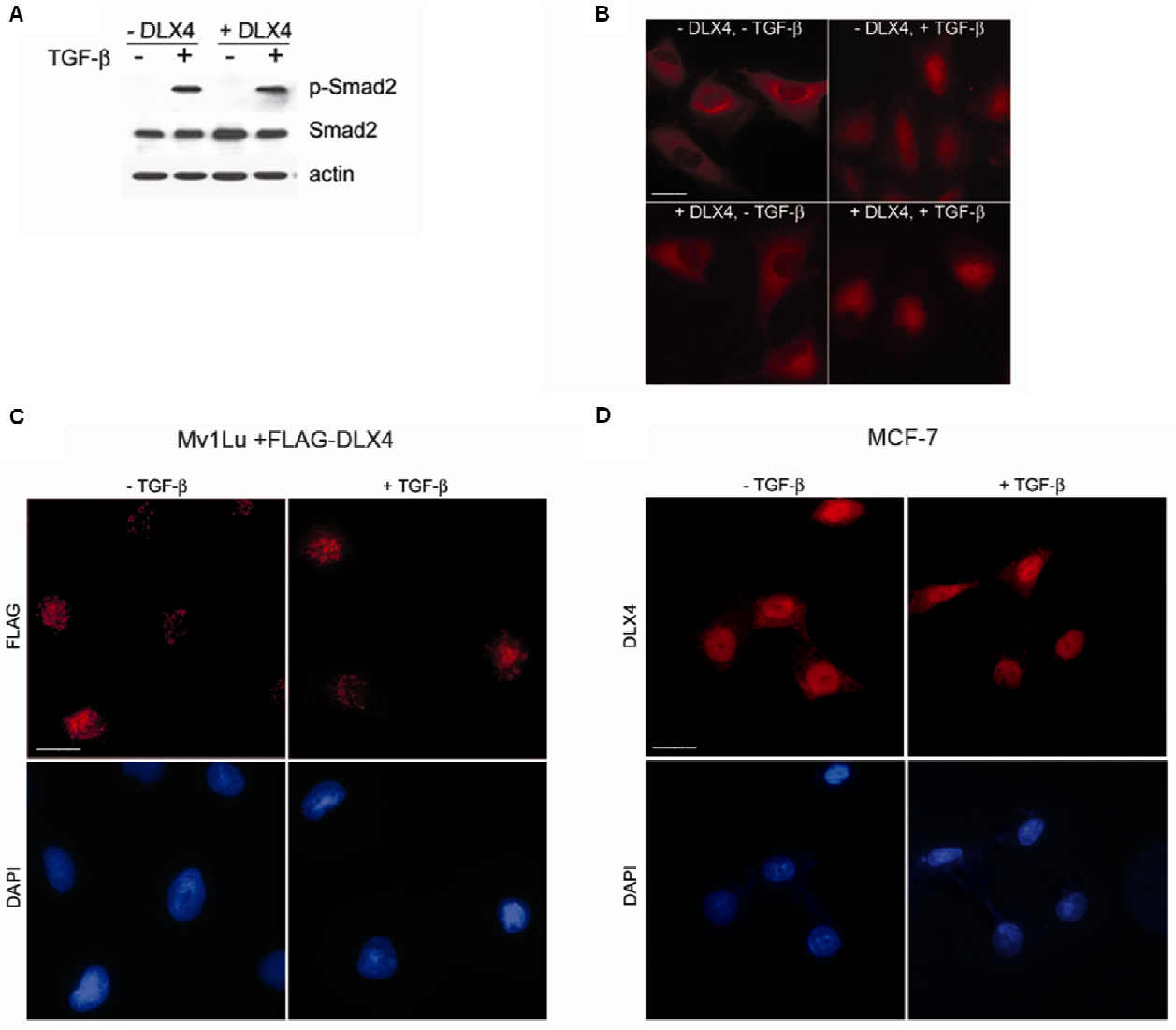
B. RESULTS

1. DLX4 does not affect phosphorylation, expression and localization of R-Smads

As discussed in Chapter 1, Smad-dependent transcription is controlled at multiple levels, including phosphorylation, expression and nuclear localization of Smad proteins (201). Enforced expression of DLX4 in Mv1Lu cells did not alter the expression level of Smad2 (Figure 22A). The expression levels of Smad3 and Smad4 were also not affected by DLX4 (see Figure 27B, discussed later). Furthermore, DLX4 did not affect TGF-β-induced phosphorylation of Smad2 (Figure 22A). This result also implies that DLX4 has no effect on activation of TGF-β receptors. Treatment of vector-control cells with TGF-β induced rapid translocation of Smad2 from the cytoplasm to the nucleus (Figure 22B). Enforced expression of DLX4 did not interfere with this translocation of Smad2 (Figure 22B). We also investigated whether TGF-β stimulation affected the localization of DLX4. Immunofluorescence staining studies demonstrated that DLX4 is predominantly localized in the nucleus and that its nuclear localization is not affected by TGF-β stimulation (Figures 22C, 22D). These findings indicate that DLX4 most likely inhibits nuclear events downstream of the TGF-β signaling pathway.

Figure 22. Phosphorylation, expression levels and localization of Smad and DLX4 proteins

Vector-control (-DLX4) and +DLX4 Mv1Lu lines were serum-starved overnight and then treated without and with TGF-β (10 ng/ml) for 30 minutes. **[A]** Total and phosphorylated Smad2 were detected by Western blot. **[B]** Intracellular localization of Smad2 was detected by immunofluorescence staining. Bar, 20 μm. **[C]** MvL1Lu cells that stably express FLAG-DLX4 were serum-starved overnight, and then incubated in complete medium for 30 minutes without or with TGF-β (10 ng/ml). Following fixation and permeabilization, cells were stained with FLAG Ab (red). **[D]** Parental MCF-7 cells were treated as in [C]. Endogenous DLX4 in cells was detected by staining using DLX4 Ab (red). Nuclei were visualized by staining with DAPI (blue). Bar, 20 μm.


A
- DLX4
+ DLX4
TGF-β
- + - +
p-Smad2
Smad2
actin
B
- DLX4, - TGF-β
- DLX4, + TGF-β
+ DLX4, - TGF-β
+ DLX4, + TGF-β
C
Mv1Lu +FLAG-DLX4
- TGF-β
+ TGF-β
FLAG
DAPI
D
MCF-7
- TGF-β
+ TGF-β
DLX4
DAPI

2. DLX4 blocks transcriptional activity of activated R-Smads

Smad proteins contain two functional domains with an intervening linker region. MH1 domains are responsible for binding to SBEs, whereas MH2 domains mediate interactions of Smads with one another and with other transcription factors (8, 12) (Figure 3). MH2 domains of Smad2 and Smad3 have intrinsic transcriptional activation capacity (8). We, therefore, sought to determine whether DLX4 inhibits transcriptional activity of Smad2 and Smad3 (Figure 23).

2.1. DLX4 represses transcriptional activity of Smad2

A chimeric expression construct was generated by fusing the GAL4 DNA-binding domain (DBD) to the linker region and MH2 transcriptional activation domain of Smad2 [amino acids 173 to 467]. To determine whether DLX4 represses transcriptional activity of Smad2, the GAL4-DBD/Smad2 chimera was co-expressed in Mv1Lu cells along with a firefly luciferase (F-Luc) reporter controlled by a synthetic promoter comprising five tandem GAL4 binding sites. As shown in Figure 24A, transcriptional activity of GAL4-Smad2 was induced by TGF-β in the absence of DLX4. However, this activation was abolished when DLX4 was expressed (Figure 24A). Similar results were obtained using the hepatoma cell line HepG2, which is responsive to TGF-β (Figure 24A). In converse experiments, we observed that knockdown of DLX4 in MCF-7 cells increased TGF-β-mediated induction of GAL4-Smad2 activity (Figures 24B).

Figure 23. Schematic design of GAL4-Smad2/3 chimeras and reporter assay

Chimeras were constructed in which the linker and MH2 domains of Smad2 and Smad3 were fused to the GAL4-DBD. These chimeras were tested for their ability to induce a synthetic promoter containing GAL4 binding element (GAL4BE).

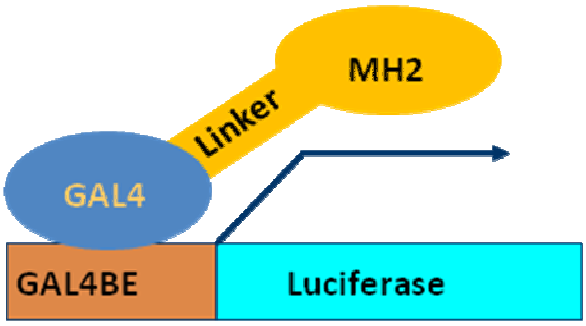
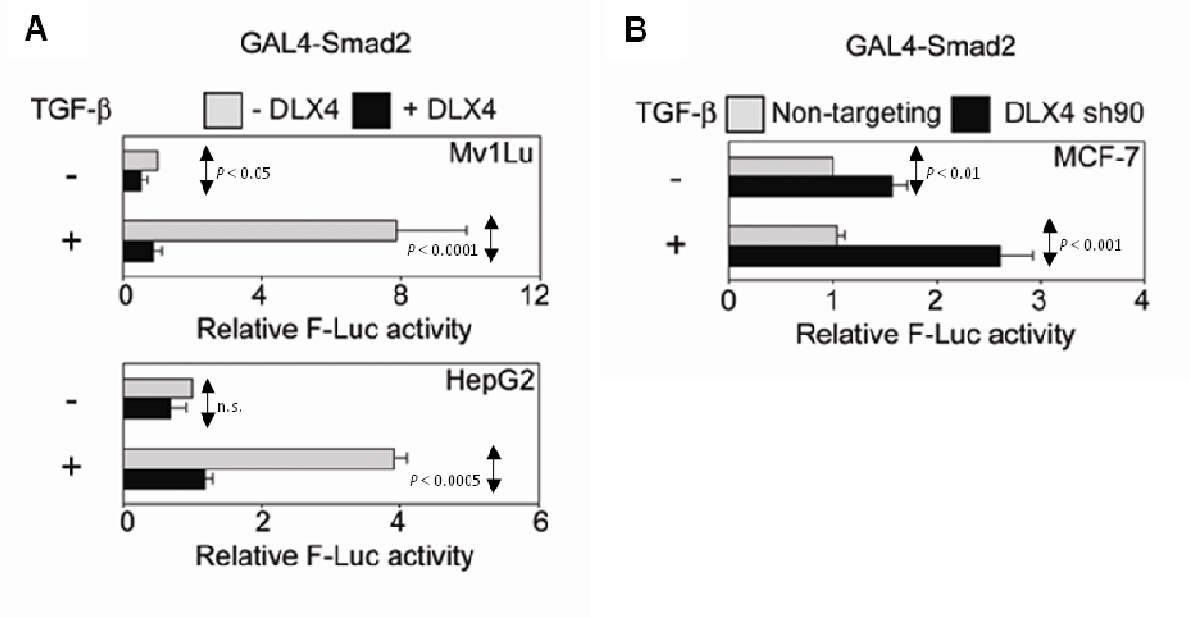


Figure 24. DLX4 blocks Smad2 transcriptional activity

[A] 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 GAL4-Smad2. Transfected cells were cultured without and with TGF-β for 18 hours, and assayed for F-Luc activity. **[B]** GAL4-Smad2 activity were likewise assayed in MCF-7 cells that were co-transfected with non-targeting shRNA (grey bar) or *DLX4* (sh90) shRNA (black bar).



2.2. DLX4 represses transcriptional activity of Smad3

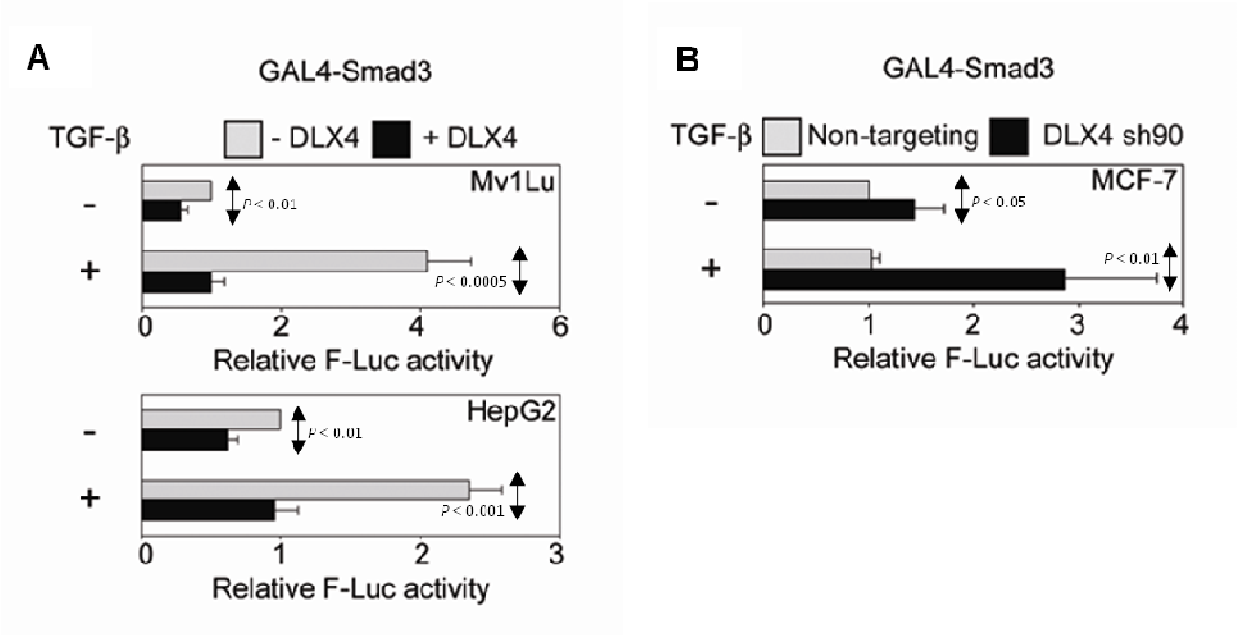
We also investigated the effect of DLX4 on transcriptional activity of Smad3 by generating a chimeric construct in which the GAL4-DBD was fused to the linker region and MH2 transcriptional activation domain of Smad3 [amino acids 133 to 425]. As observed with our assays using the GAL4-Smad2 chimera, we found that DLX4 similarly abolished TGF-β-induced transcriptional activity of the GAL4-Smad3 chimera in both Mv1Lu and HepG2 cells (Figure 25A). Conversely, knockdown of DLX4 in MCF-7 cells enhanced TGF-β-mediated induction of GAL4-Smad3 activity (Figure 25B).

2.3. DLX4 blocks gene transcription mediated by BMP-activated R-Smads

Smad2 and Smad3 serve as substrates for the TβRI kinase and are activated by TGF-β, whereas other R-Smads (Smads 1, 5 and 8) are utilized by the BMP and anti-Müllerian receptors (10, 201). 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). Activity of this promoter was induced by TGF-β treatment of vector-control HepG2 cells (Figure 26A). This induction was blocked by DLX4. Similarly, BMP-4 treatment of HepG2 cells induced activity of the SBE-driven promoter, whereas DLX4 blocked BMP-4-mediated induction of the promoter (Figure 26A). TGF-β- and BMP-specific R-Smads have been reported to preferentially bind distinct DNA sequences (196, 202). Indeed, we found that BMP-4 was not as effective as TGF-β in inducing pSBE4-Luc activity (Figure 26A). We,

Figure 25. DLX4 blocks Smad3 transcriptional activity

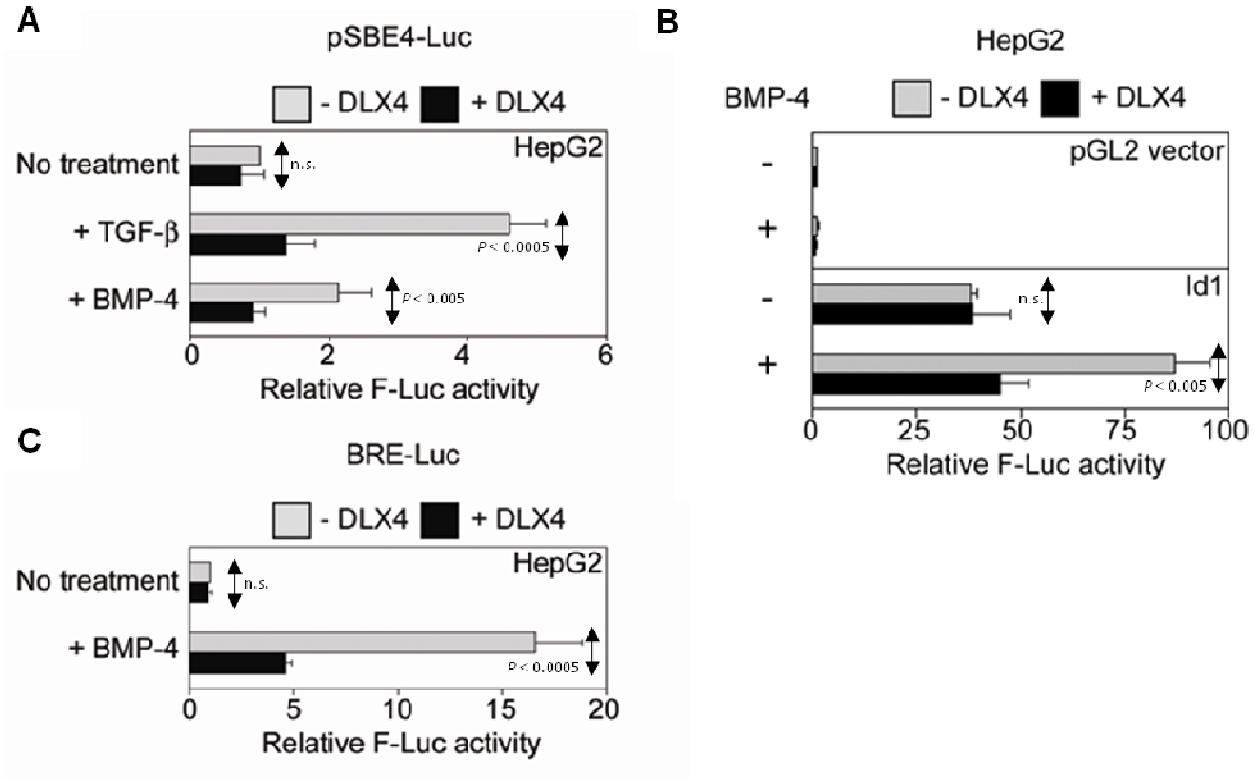
[A] Mv1Lu and HepG2 cells were co-transfected with empty vector (grey bar) or DLX4 (black bar), together with GAL4-driven F-Luc reporter plasmid and with GAL4-Smad3. Transfected cells were cultured without and with TGF-β for 18 hours, and assayed for F-Luc activity. **[B]** GAL4-Smad3 activities were, likewise, assayed in MCF-7 cells that were co-transfected with non-targeting shRNA (grey bar) or *DLX4* (sh90) shRNA (black bar).



therefore, tested the effect of DLX4 on BMP-induced transcription by using a reporter plasmid that contains the BMP-responsive promoter of the *Id1* gene. BMP-4 induced *Id1* promoter activity in vector-control HepG2 cells, whereas expression of DLX4 blocked BMP-induced *Id1* promoter activity (Figure 26B). To confirm this blocking effect of DLX4, we used a synthetic promoter comprising two tandem copies of the BMP response elements of the *Id1* promoter (BRE-Luc). Activity of this promoter was induced by BMP-4 treatment of vector-control HepG2 cells. However, BMP-4-induced activation of the promoter was blocked when DLX4 was expressed (Figure 26C). Because TGF-β- and BMP-specific R-Smads utilize Smad4 as the common and essential partner for the formation of functional transcriptional complexes (201), our findings raise the possibility that DLX4 inhibits Smad4.

Figure 26. Effect of DLX4 on TGF-β and BMP-induced transcription

[A] HepG2 cells were co-transfected with empty vector (grey bar) or DLX4 (black bar) together with SBE-driven pSBE4-Luc reporter plasmid, and then cultured without and with TGF-β (10ng/ml) or BMP-4 (80 ng/ml) for 18 hours. **[B]** HepG2 cells were co-transfected with empty vector (grey bar) or DLX4 (black bar), together with reporter plasmids containing no promoter (pGL2 vector) or a 1.6 kb region of the *Id1* promoter. At 24 hours after transfection, cells were cultured without and with BMP-4 (80 ng/ml) for additional 18 hours. **[C]** HepG2 cells were co-transfected with empty vector (grey bar) or DLX4 (black bar) together with BRE-Luc reporter plasmid, and then cultured without and with BMP-4 (80 ng/ml) for 18 hours. Shown are relative F-Luc activities in three independent experiments each performed in duplicate and normalized by activity of co-transfected R-Luc.



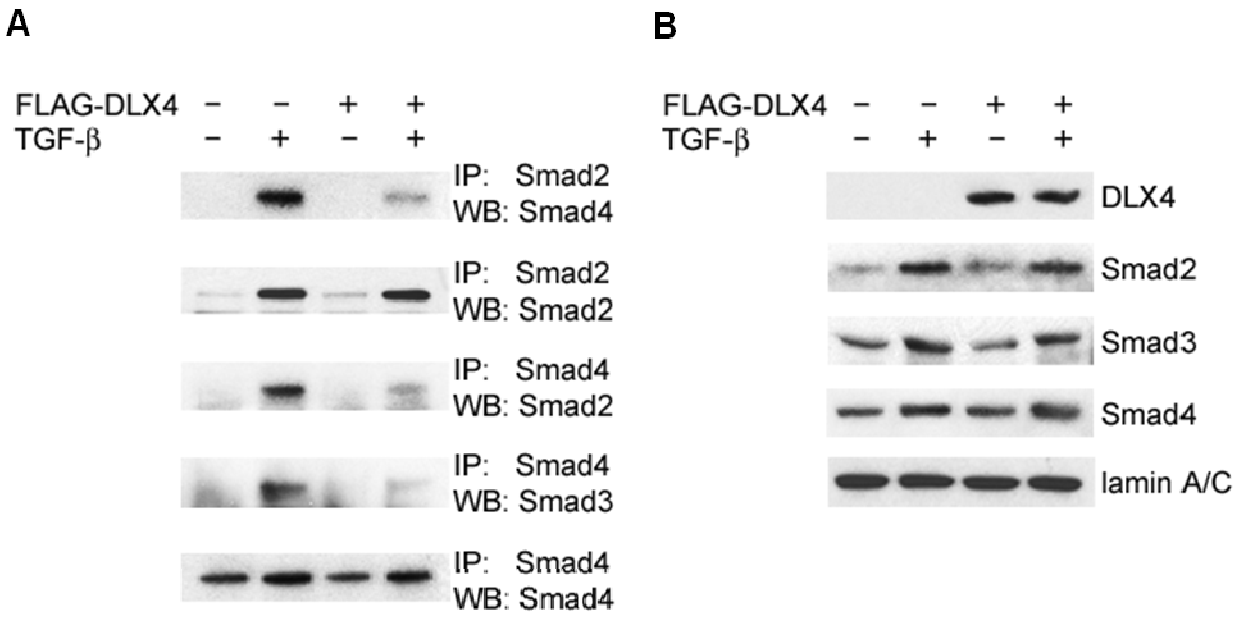
3. DLX4 prevents Smad4 from binding R-Smads

The formation of R-Smad/Smad4 transcriptional complexes is essential for Smad-mediated transcription (10, 12). In subsequent experiments, we 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 were analyzed by immunoblotting using Ab to Smad4. Binding of Smad4 to Smad2 was observed following TGF-β treatment. However, this interaction was inhibited when DLX4 was expressed (Figure 27A). Identical results were obtained in reciprocal immunoprecipitation (IP) assays in which Smad4 was immunoprecipitated and Smad2 was detected in precipitates (Figure 27A).

Because Smad2 and Smad3 are highly homologous and they both interact with Smad4 via their MH2 domains (8, 203), we determined whether DLX4 also interferes with interaction of Smad3 and Smad4. As shown in Figure 27A, treatment of vector-control HepG2 cells with TGF-β induced binding of Smad4 to Smad3. In contrast, this binding was inhibited when DLX4 was expressed. DLX4 did not alter expression levels of Smad2, Smad3 or Smad4 (Figure 27B). Together, these results indicate that DLX4 likely inhibits transcriptional activity of Smad2 and Smad3 by preventing Smad4 from interacting with these R-Smads.

Figure 27. DLX4 prevents Smad4 from binding R-Smads

HepG2 cells were transfected with empty vector or with FLAG-tagged DLX4. At 24 hours thereafter, cells were serum-starved overnight and then treated without and with TGF-β (10ng/ml) for 30 minutes. **[A]** Smad2 was immunoprecipitated from nuclear extracts and precipitates were 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 and Smad proteins in nuclear extracts.



4. DLX4 interacts with Smad4

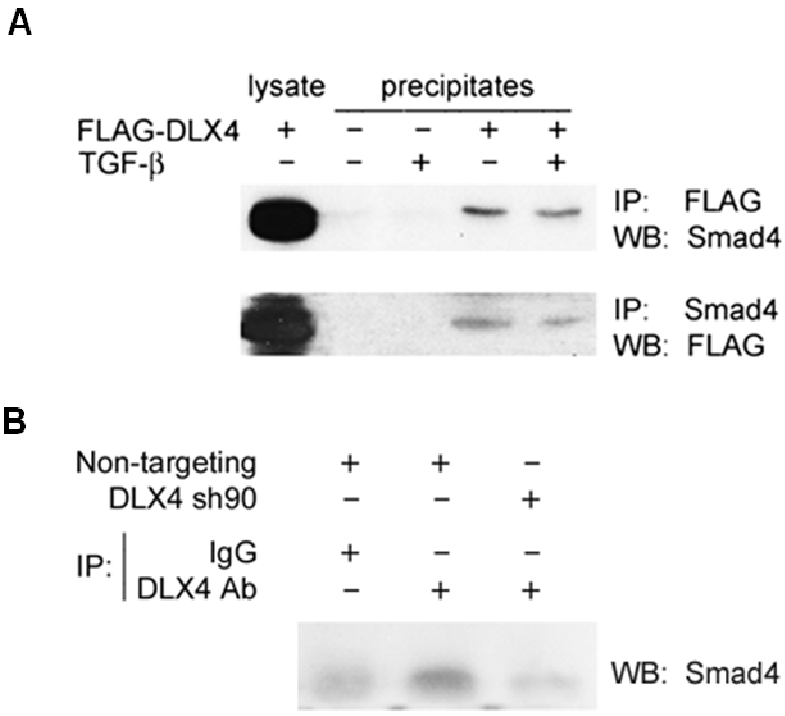
Our findings that DLX4 prevents Smad4 from interacting with R-Smads raise the possibility that DLX4 binds Smad4. In subsequent experiments, we determined whether DLX4 interacts with Smad4 and whether this interaction involves direct binding.

4.1. 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 or empty vector. We found that FLAG-tagged DLX4 associated with Smad4, irrespective of TGF-β stimulation (Figure 28A). We next determined whether endogenous DLX4 could interact with Smad4. Endogenous DLX4 was immunoprecipitated by DLX4 Ab from lysates of MCF-7 cells, and Smad4 was detected in precipitates. IP of normal IgG was included as a negative control (Figure 28B). To additionally confirm the interaction of DLX4 with Smad4, we performed IP using extracts of MCF-7 cells that had been transfected with *DLX4* shRNA (sh90) or with non-targeting shRNA. As shown in Figure 28B, the interaction of DLX4 with Smad4 was reduced when *DLX4* was knocked-down. 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 28C). DLX4 also bound Smad2, but this binding was weaker than binding to Smad4 (Figure 28C).

Figure 28. DLX4 binds to Smad4

[A] HepG2 cells were transfected with empty vector or with FLAG-tagged DLX4. At 24 hours 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)*.


A
lysate
precipitates
FLAG-DLX4 + − − + +
TGF-β − − + − +
IP: FLAG
WB: Smad4
IP: Smad4
WB: FLAG
B
Non-targeting + + −
DLX4 sh90 − − +
IP: IgG + − −
DLX4 Ab − + +
WB: Smad4
C
GST
GST-Smad2
GST-Smad4
kD
75 -
50 -
37 -
DLX4-FL Input (20%)
GST
GST-Smad2
GST-Smad4
kD
37 -
25 -

4.2. Identification of binding domains of DLX4 and Smad4

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 full-length Smad4 (Figure 29A). We generated GST-DLX4 fusion proteins that contained full-length DLX4 (FL), only the homeodomain (HD), only the transactivation domain (TA), and the transactivation domain and homeodomain but lacking the C-terminal tail (TA+HD) (Figure 29B). Deletion of the C-terminal tail of DLX4 (TA+HD) only weakly affected its ability to bind Smad4 (Figure 29C). In contrast, deletion of the DNA-binding homeodomain of DLX4 markedly inhibited its Smad4-binding ability (TA). Binding of the DLX4 homeodomain to Smad4 (HD) was detected but not as strongly as observed with full-length DLX4 (Figure 29C).

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 30A). We generated *in vitro*-translated proteins that contained full-length Smad4 (FL), only the MH1 domain (MH1), only the MH2 domain (MH2), and the MH1 and MH2 domains with the linker region (MH1+LK, LK+MH2) (Figure 30A). Direct binding of DLX4 was detected to the Smad4 MH1 domain alone, but not to the MH2 domain (Figure 30C).

Figure 29. DLX4 binds to Smad4 in part via the homeodomain

[A] GST-DLX4 constructs comprising the transactivation domain (TA), homeodomain (HD) and C-terminal tail (C). **[B]** Full-length (FL) DLX4 and portions thereof were expressed as GST-fusion proteins, and **[C]** assayed for binding to ^{35}S-labeled full-length Smad4.

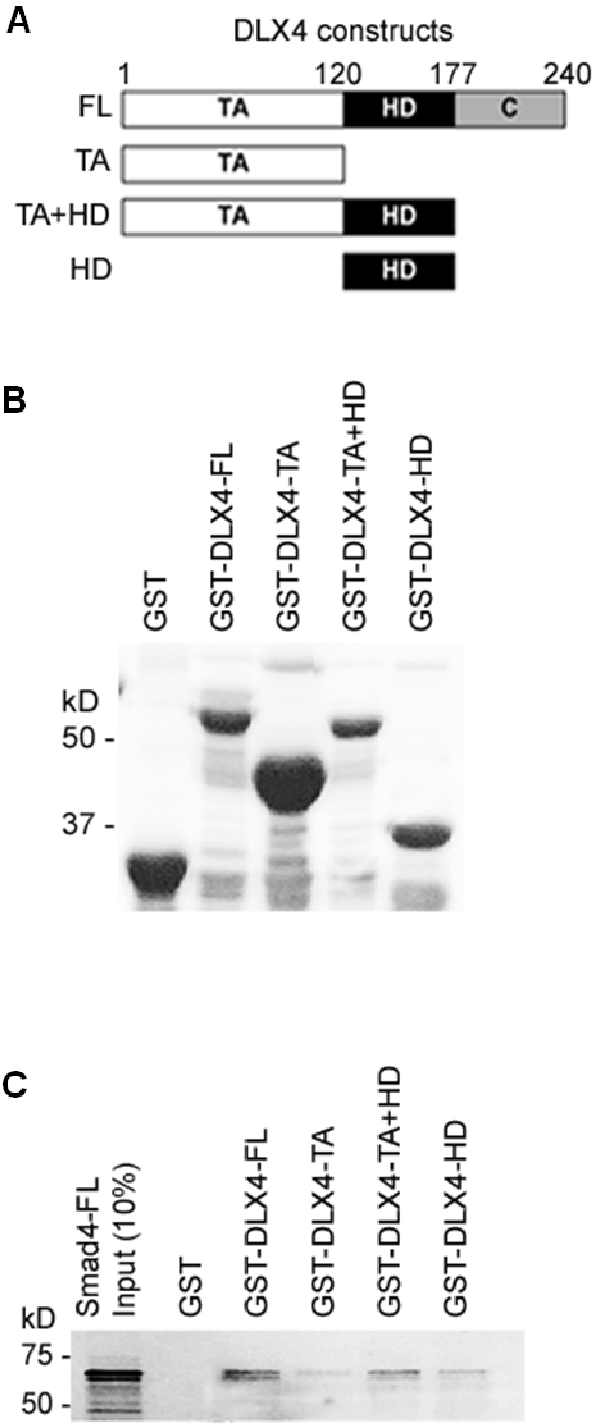
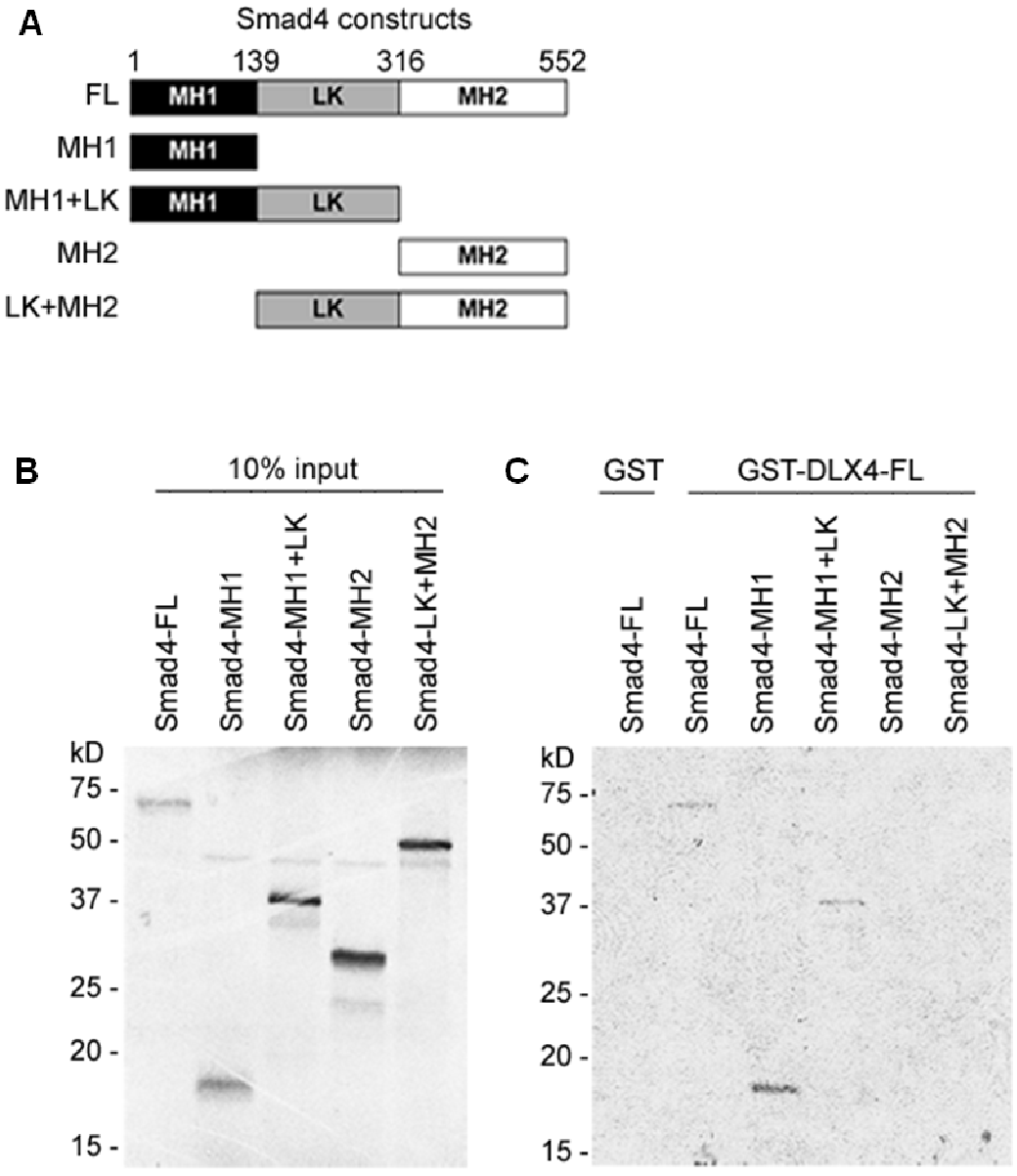

A
DLX4 constructs
1
120
177
240
FL
TA
HD
C
TA
TA
TA+HD
TA
HD
HD
B
GST
GST-DLX4-FL
GST-DLX4-TA
GST-DLX4-TA+HD
GST-DLX4-HD
kD
50 -
37 -
C
Smad4-FL
Input (10%)
GST
GST-DLX4-FL
GST-DLX4-TA
GST-DLX4-TA+HD
GST-DLX4-HD
kD
75 -
50 -

Figure 30. DLX4 binds to the MH1 domain of Smad4

[A] Smad4 constructs comprising MH1 and MH2 domains and linker (LK) region. **[B]** ^{35}S-labeled full-length and truncated Smad4 were translated *in vitro* and **[C]** assayed for binding to full-length GST-DLX4 protein.



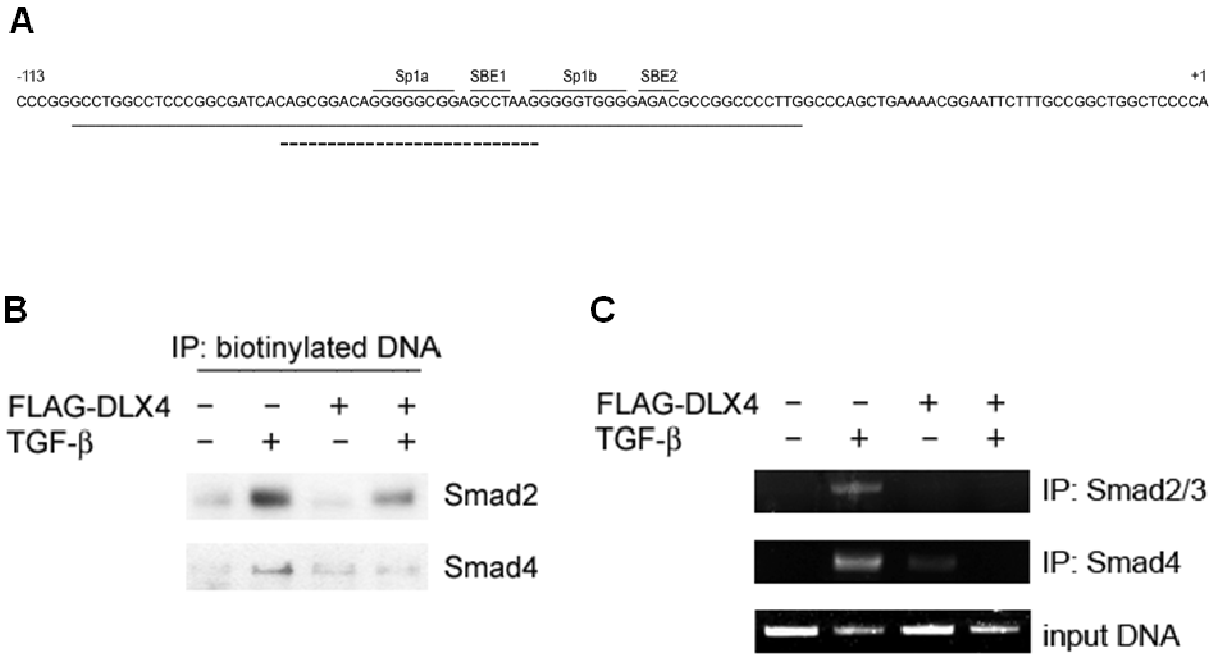
5. DLX4 blocks interaction of Smad proteins with DNA

Smad proteins bind to SBEs via their MH1 domains (33). Because DLX4 binds to the MH1 domain of Smad4, we investigated whether DLX4 blocks the interaction of Smad proteins to DNA. This was initially investigated by *in vitro* DNA pull-down assays. A biotinylated oligonucleotide containing sequences of the minimal $p15^{Ink4B}$ promoter region (nucleotides -108 to -39), including the SBEs (Figure 31A), was used to pull-down Smad proteins from nuclear extracts. Increased levels of Smad2 and Smad4 were detected in DNA-protein complexes when vector-control HepG2 cells were stimulated with TGF-β (Figure 31B). In contrast, these increased levels of Smad interactions with DNA were not observed when DLX4 was expressed (Figure 31B).

To confirm the ability of DLX4 to block Smad-DNA interactions in a more physiological context, we performed ChIP assays. As shown in Figure 31C, association of Smad4 and R-Smads with the $p15^{Ink4B}$ promoter was detected by ChIP assays in vector-control HepG2 cells following TGF-β treatment. In contrast, interactions of Smad proteins with the $p15^{Ink4B}$ promoter were abrogated when DLX4 was expressed (Figure 31C).

Figure 31. DLX4 blocks interactions of Smad proteins with the p15^{Ink4B} promoter

[A] Sequence of the minimal p15^{Ink4B} promoter indicating Sp1 binding sites and SBEs (adapted from (51)). Underlined are sequences contained in the oligonucleotides used for oligonucleotide pull-down assays (solid line) and gel-shift assays (dashed line). **[B]** 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. **[C]** 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.



C. CONCLUSION

The studies in this chapter provide significant insight into the mechanism by which DLX4 blocks TGF-β/Smad signaling. Firstly, we demonstrate that DLX4 does not alter phosphorylation, expression and nuclear localization of Smad proteins but that DLX4 blocks TGF-β-induced transcriptional activity of Smad2 and Smad3. Our observation that DLX4 prevents Smad4 from binding to R-Smads suggests that DLX4 inhibits the formation of R-Smad/Smad4 transcriptional complexes. We also demonstrate that DLX4 directly binds to the MH1 domain of Smad4, and that binding of DLX4 to Smad4 is mediated in part via its homeodomain. Direct binding of DLX4 to Smad4 and the ability of DLX4 to also block BMP-induced transcription suggests that DLX4 could block signaling induced by different ligands of the TGF super-family that all utilize Smad4. In addition to the binding of Smad transcriptional complexes to SBEs, the interaction of Smad proteins with other transcription factors dictates the specificity and affinity of Smad complexes for target gene promoters. Understanding the interactions of Smads with other transcription factors will be the focus of Chapter 5.

CHAPTER 5: DLX4 REPRESSES SMAD/SP1-MEDIATED TRANSCRIPTION

A. RATIONALE

Both the strength and specificity of Smad-mediated transcription are governed by interactions of Smad complexes with specific transcriptional factors and their interactions with corresponding DNA-binding elements on target promoters (8, 10, 35) (Figures 4, 5). Transcriptional activation of genes encoding the CDK inhibitors $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ is mediated by cooperative interactions between Sp1 and Smads that bind to GC boxes and SBEs respectively on the $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ promoters (36, 38, 51).

In Chapters 3 and 4, we determined that DLX4 blocks Smad-mediated induction of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$. We found that DLX4 binds to Smad4, and blocks the interaction of Smad4 with R-Smads, and also the interactions of Smad complexes with DNA. Because cooperative interactions between Sp1 and Smads are important for Smad-mediated transcription of the CDK inhibitor genes, we hypothesize that DLX4 could interfere with Smad-Sp1 interactions. The goal of the studies in this chapter is to determine whether and how DLX4 modulates Smad-Sp1-mediated transcription.

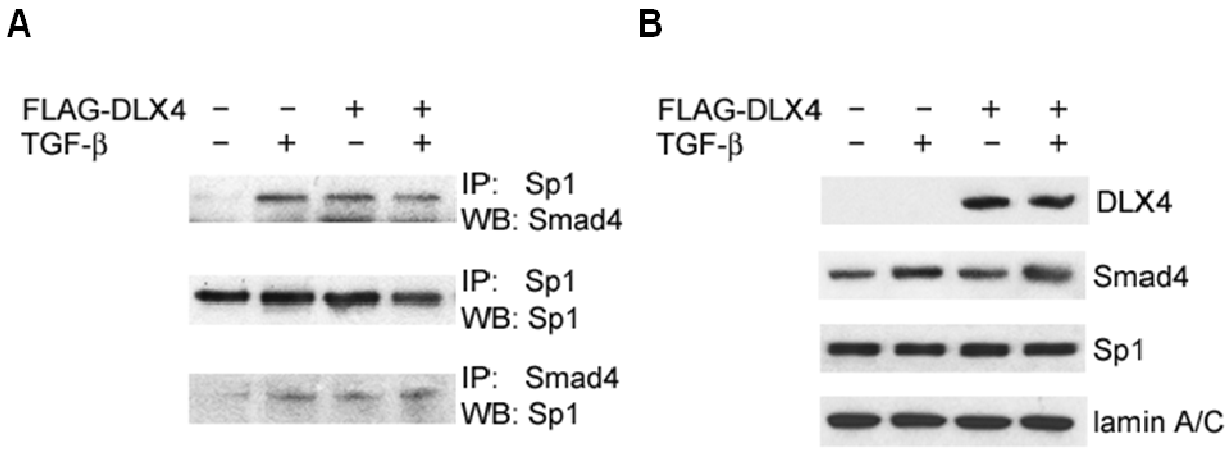
B. RESULTS

1. DLX4 does not prevent interactions of Smad4 and Sp1 proteins

TGF-β-induced transcription of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ requires cooperative interactions between Sp1 and Smads proteins, in particular, Smad4, (37, 38, 51). In initial experiments, we investigated whether DLX4 interferes with the interaction of Smad4 with Sp1 by IP assays. Following transfection with FLAG-tagged DLX4 or empty vector, HepG2 cells were treated with or without TGF-β. Sp1 was immunoprecipitated, and precipitates were analyzed by immunoblotting using Ab to Smad4. As reported in other studies (51), we similarly observed binding of Smad4 to Sp1 following TGF-β treatment of vector-control cells. Surprisingly, we observed interaction of Smad4 with Sp1 in cells that expressed DLX4, even in the absence of TGF-β stimulation (Figure 32A). These observations were confirmed in reciprocal IP assays in which Smad4 was immunoprecipitated and Sp1 was assayed in precipitates (Figure 32A). DLX4 did not alter the expression levels of Sp1 and Smad4 (Figure 32B). This finding indicates that DLX4 facilitates interaction between Smad4 and Sp1 independently of TGF-β stimulation.

Figure 32. DLX4 facilitates interactions of Smad4 and Sp1

HepG2 cells were transfected with empty vector or with FLAG-tagged DLX4. At 24 hours thereafter, cells were serum-starved overnight and then treated without and with TGF-β (10 ng/ml) for 30 minutes. **[A]** Sp1 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 Sp1 Ab. **[B]** Western blot of DLX4 and Smad4 and Sp1 in nuclear extracts.



2. DLX4 interacts with Sp1

In Smad/Sp1 transcriptional complexes, Sp1 directly binds Smad4 (51). Because we previously found that DLX4 directly binds Smad4, it is possible that DLX4 also interacts with Sp1. In subsequent experiments, we determined i) whether DLX4 interacts with Sp1, ii) whether this interaction involves direct binding, and iii) what are the binding domains of Sp1 and DLX4.

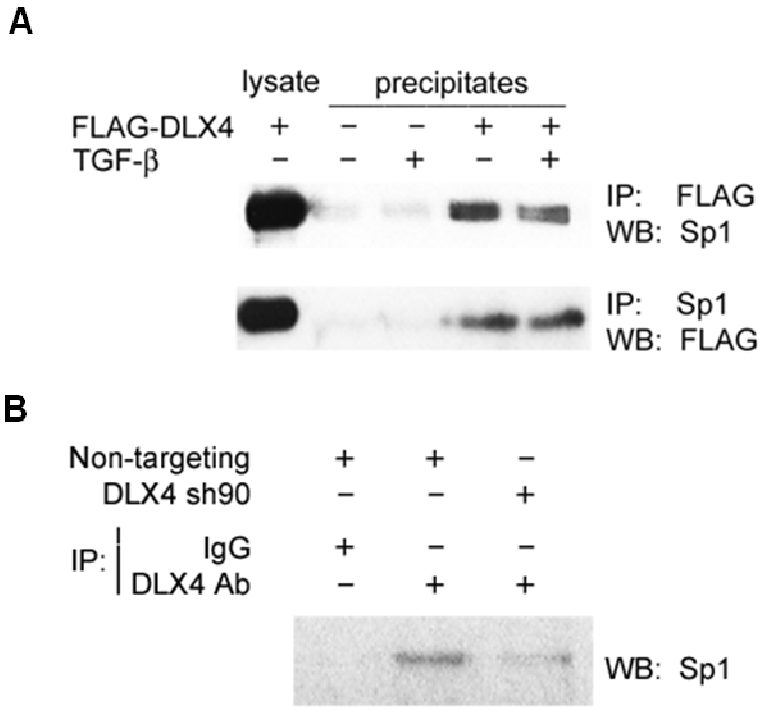
2.1. DLX4 directly binds Sp1

We first determined whether DLX4 interacts with Sp1 in cells. IP assays were performed using extracts of HepG2 cells that expressed FLAG-DLX4 or empty vector. FLAG-tagged DLX4 associated with Sp1, irrespective of TGF-β stimulation (Figure 33A). We next determined whether endogenous DLX4 interacts with Sp1. Endogenous DLX4 was immunoprecipitated by DLX4 Ab from lysates of MCF-7 cells, and Sp1 was detected in precipitates. IP of normal IgG was included as a negative control (Figure 33B). To confirm the interaction of DLX4 with Sp1, we performed IP using lysates of MCF-7 cells that had been transfected with *DLX4* shRNA (sh90) or with non-targeting shRNA. As shown in Figure 33B, the interaction of DLX4 with Sp1 was reduced when *DLX4* was knocked-down.

To determine whether DLX4 directly binds Sp1, we performed GST-pulldown assays. Full-length GST-DLX4 fusion protein (FL) directly bound to *in vitro*-translated

Figure 33. DLX4 associates with Sp1

[A] Lysates were prepared from HepG2 cells as described in Figure 32A. 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.



^{35}S-labeled full-length Sp1 (Figure 34A). Similarly, full-length GST-Sp1 fusion protein was observed to bind *in vitro*-translated ^{35}S-labeled DLX4 (Figure 34C).

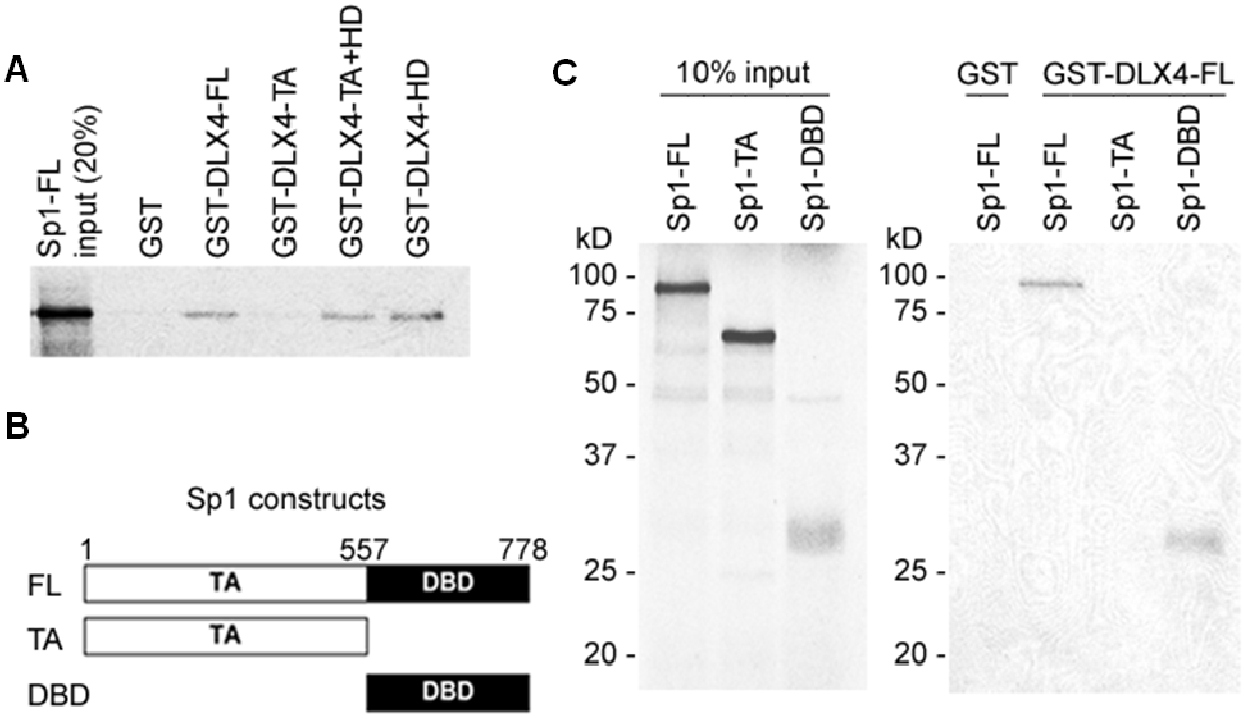
2.2. Identification of binding domains of DLX4 and Sp1

We determined the domain of DLX4 that binds to Sp1 by performing GST pull-down assays using GST-DLX4 fusion proteins that contained different domains of DLX4 (Figure 29A, B). Binding of the homeodomain of DLX4 to *in vitro*-translated Sp1 was strongly detected (Figure 34A). This binding was comparable to binding of full-length DLX4 to Sp1. Deletion of the C-terminal tail or the transactivation domain of DLX4 did not affect its ability to bind Sp1 (Figure 34A).

In converse experiments, we determined the domain of Sp1 that binds DLX4. Sp1 comprises a C-terminal DNA-binding domain [amino acids 557 to 778] and an N-terminal transactivation domain [amino acids 1 to 557] (Figure 34B). We generated *in vitro*-translated ^{35}S-labeled proteins that contained full-length Sp1 (FL), the DNA-binding domain of Sp1 (DBD) and its transactivation domain (TA) (Figure 34C). DLX4 did not bind the transactivation domain of Sp1, but bound to its DNA-binding domain (Figure 34C). Together, these findings demonstrate that the interaction between DLX4 and Sp1 is mediated via their respective DNA-binding domains.

Figure 34. DLX4 binds to the DNA-binding domain of Sp1

[A] GST-DLX4 proteins (described in Figure 29A) were assayed for binding to ^{35}S-labeled full-length Sp1. **[B]** Sp1 constructs comprising the transactivation domain (TA) and DNA-binding domain (DBD). **[C]** ^{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)*.



3. DLX4 inhibits DNA-binding activity of Sp1

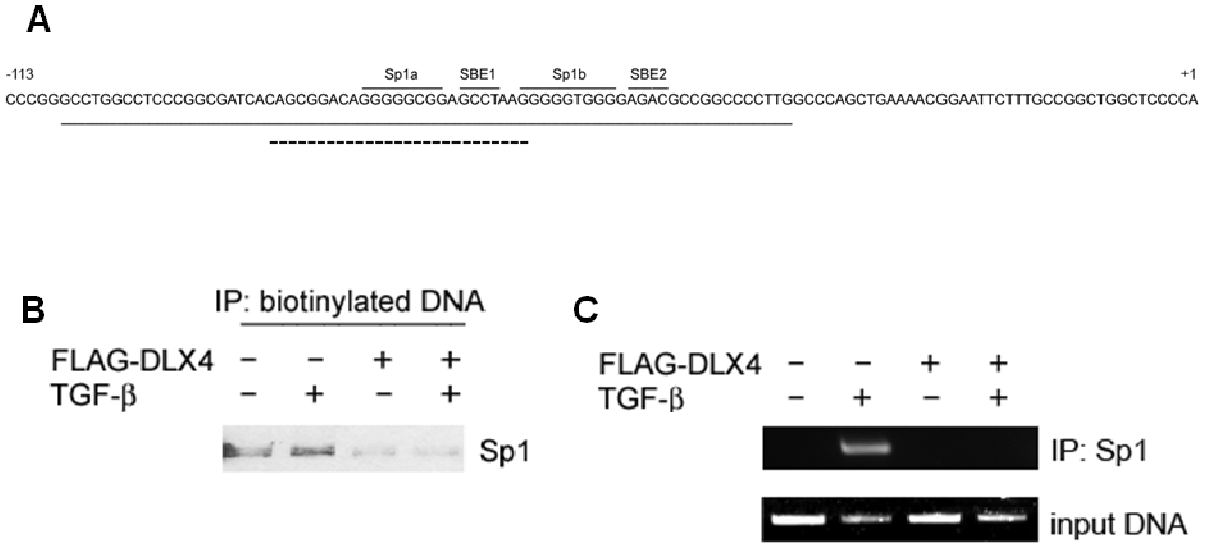
3.1. DLX4 blocks interactions of Sp1 with the p15^{Ink4B} promoter

Because DLX4 binds the DNA-binding domain of Sp1, it is possible that DLX4 interferes with the DNA-binding ability of Sp1. To determine whether DLX4 alters the binding of Sp1 to its recognition element, we performed *in vitro* DNA pull-down assays using a biotinylated oligonucleotide containing sequences of the minimal p15^{Ink4B} promoter region (nucleotides -108 to -39). This contains two GC boxes plus two SBEs (Figure 35A). In contrast to Smad proteins, binding of Sp1 to this promoter was detected in lysates of vector-control HepG2 cells in the absence of TGF-β stimulation (Figure 35B). TGF-β stimulation increased the level of DNA-bound Sp1 in vector-control cells (Figure 35B). However, very little DNA-bound Sp1 was detected in lysates of HepG2 cells that expressed DLX4, irrespective of TGF-β stimulation (Figure 35B).

To confirm this finding in a more physiological context, ChIP was performed to assay binding of endogenous Sp1 to the p15^{Ink4B} promoter. As shown in Figure 35C, association of Sp1 with the p15^{Ink4B} promoter was detected in vector-control HepG2 cells following TGF-β treatment. In contrast, binding of Sp1 to the p15^{Ink4B} promoter was not detected in cells that expressed DLX4, irrespective of TGF-β stimulation (Figure 35C). The ability of DLX4 to block binding of Sp1 to the p15^{Ink4B} promoter is similar to its ability to block DNA-binding of Smad proteins as we demonstrated in Chapter 4. Together, these findings indicate that DLX4 blocks the interaction of Smad/Sp1 complexes with target gene promoters.

Figure 35. DLX4 blocks interactions of Sp1 protein with the p15^{Ink4B} promoter

[A] Sequence of the minimal p15^{Ink4B} promoter indicating Sp1 binding sites and SBEs (adapted from (51)). Underlined are sequences contained in the oligonucleotides used for oligonucleotide pull-down assays (solid line) and gel-shift assays (dashed line). **[B]** 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. **[C]** ChIP analysis of interactions of Sp1 with the *p15^{Ink4B}* promoter. The input fraction corresponded to 1 % of the chromatin solution of each sample before IP.



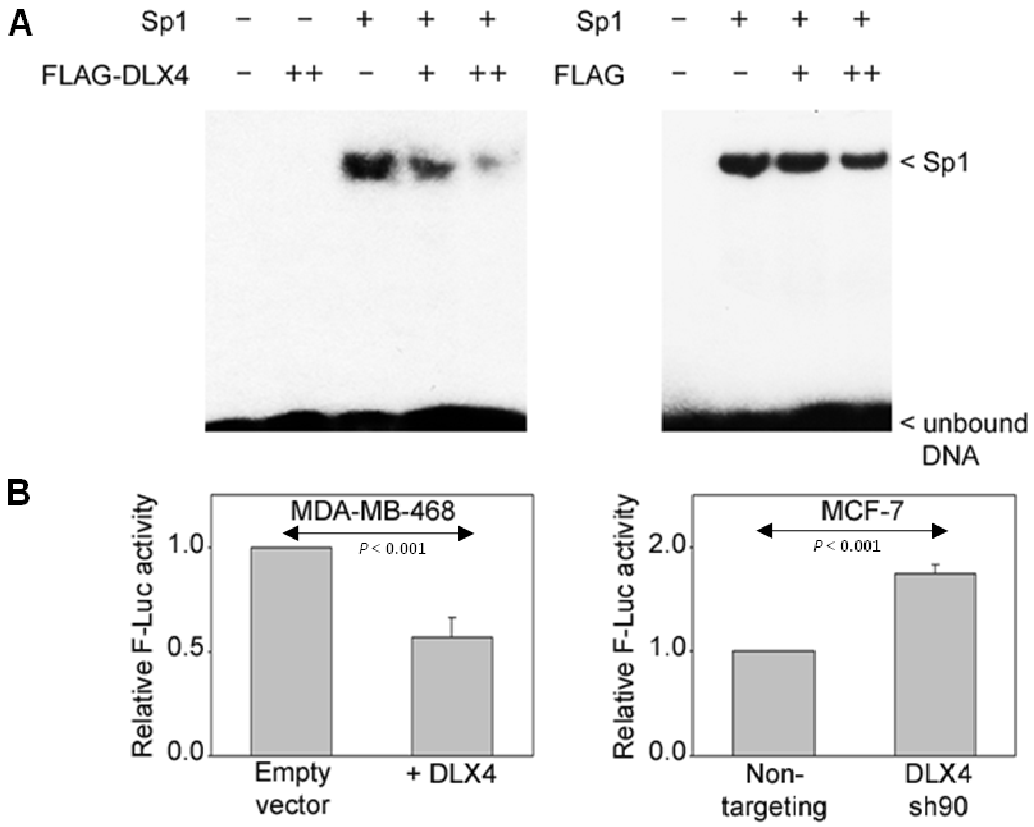
3.2. DLX4 blocks the DNA-binding ability of Sp1, independently of Smad proteins

In subsequent experiments, we sought to determine whether DLX4 blocks the DNA-binding ability of Sp1, independently of Smad proteins. Gel-shift assays were performed to test the ability of DLX4 to prevent Sp1 from binding sequences of the minimal $p15^{Ink4B}$ promoter region (nucleotides -88 to -63). Addition of increasing amounts of recombinant FLAG-tagged DLX4 blocked the binding of recombinant Sp1 to the DNA probe (Figure 36A). In contrast, addition of increasing amounts of FLAG-tag alone did not affect the DNA-binding ability of Sp1 (Figure 36A).

Because DLX4 blocks DNA-binding ability of Sp1, we also investigated whether DLX4 blocks Sp1-induced transcription, independently of Smads. We performed reporter assays using a synthetic promoter that comprised tandem Sp1-binding sites (Figure 36B). Enforced expression of DLX4 in Smad4-deficient MDA-MB-468 cells inhibited activity of the Sp1-driven promoter (Figure 36B). Conversely, knock-down of DLX4 in MCF-7 cells stimulated Sp1-driven promoter activity (Figure 36B).

Figure 36. DLX4 blocks DNA-binding ability of Sp1

[A] Gel shift analysis using a ^{32}P-labeled oligonucleotide containing nucleotides -88 to -64 of the p15^{Ink4B} promoter (refer figure 35A). Recombinant Sp1 protein was incubated with increasing amounts of *in vitro* translated FLAG-DLX4 and FLAG-tag. Gel-shifted DNA-bound Sp1 is indicated. **[B]** 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.



C. CONCLUSION

Cooperative interactions between Smad proteins and Sp1 are important for TGF-β-induced transcription of CDK inhibitor genes. In this chapter, we identified the mechanism of how DLX4 blocks Smad/Sp1-mediated transcription. We found that DLX4 does not prevent the interaction between Sp1 and Smad4, but blocks the interactions of Sp1 and Smad proteins with the $p15^{Ink4B}$ promoter. We also demonstrate that DLX4 directly binds to the DNA-binding domain of Sp1, and inhibits the DNA-binding ability of Sp1. Together, these results indicate that DLX4 inhibits Smad/Sp1-mediated transcription by inhibiting the DNA-binding ability of Sp1, in addition to preventing Smad4 from interacting with R-Smads as identified in Chapter 4.

CHAPTER 6: DISCUSSION

A. DLX4 COUNTERACTS KEY TRANSCRIPTIONAL CONTROL MECHANISMS OF THE TGF-β CYTOSTATIC PROGRAM

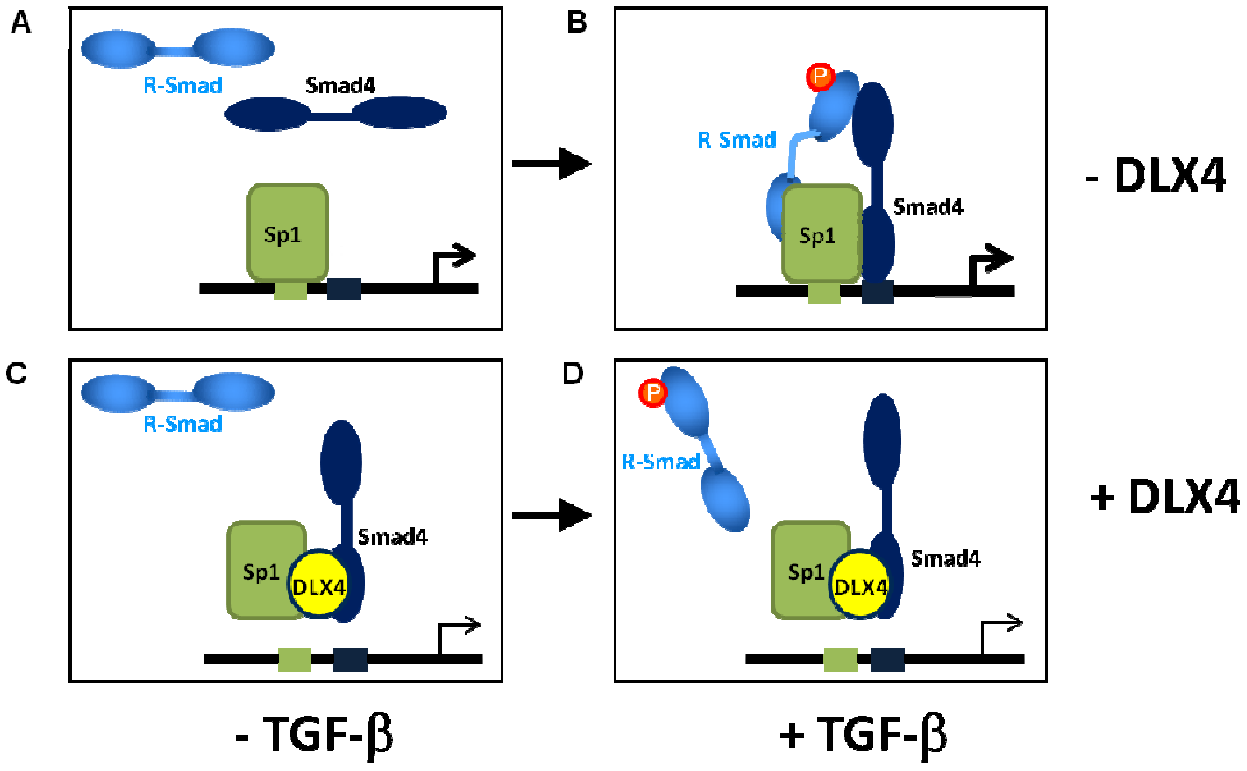
The TGF-β cytostatic program is essential for maintaining normal tissue homeostasis. Gene responses that are central to the TGF-β cytostatic program include induction of the CDK inhibitors $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ and repression of c-*myc*. These gene responses are tightly regulated by a repertoire of transcriptional regulators that include Smad proteins, Sp1 and c-myc (3, 4). My studies in Chapter 3 (Aim 1) demonstrate that DLX4, a homeodomain protein that is expressed in many types of cancers, blocks the anti-proliferative effect of TGF-β by preventing G_1 arrest. DLX4 inhibits TGF-β-induced expression of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ and blocks TGF-β-mediated repression of c-myc expression. In addition, my studies demonstrate that DLX4 induces c-myc expression independently of TGF-β signaling. My studies in Chapter 4 (Aim 2) and Chapter 5 (Aim 3) identify and characterize several distinct mechanisms by which DLX4 inactivates transcriptional control of the TGF-β cytostatic program.

1. DLX4 sequesters Smad4 and prevents Smad4 from binding R-Smads

The formation of transcriptional complexes of Smad4 and activated R-Smads upon TGF-β stimulation is essential for Smad-mediated transcription (10, 12). My studies indicate that DLX4 does not alter Smad phosphorylation, expression or nuclear translocation (Figure 22). However, my studies identify that DLX4 blocks Smad transcriptional activity (Figures 24 and 25) by directly binding to Smad4 and preventing Smad4 from binding to R-Smads (Figures 27 and 28). Smad interactions might also be prevented by the binding of DLX4 to R-Smads, as we observed that DLX4 directly binds to Smad2 (Figure 28). However, binding of DLX4 to Smad2 was much weaker than to Smad4. Because Smad4 and R-Smads interact with one another via their MH2 domains (201), our finding that DLX4 binds the Smad4 MH1 domain is somewhat surprising. One explanation could be that binding of DLX4 to the Smad4 MH1 domain induces a conformational change such that the MH2 domain of Smad4 is unable to interact with R-Smads (Figure 37). One way to test this would be to perform X-ray crystallography studies of DLX4 interactions with Smad proteins. However, since Smad proteins bind DNA via their MH1 domains, the binding of DLX4 to the MH1 domain is consistent with the observed ability of DLX4 to block the ability of Smads to bind DNA (Figure 31). Smad proteins have been reported to interact with a variety of other transcription factors (reviewed in (203)). However, most of these other transcription factors interact with the MH2 domain of Smad proteins, and very few interact with the MH1 domain.

Figure 37. Predicted model of DLX4, Smad4 and Sp1 interactions

In the absence of TGF-β stimulation, Sp1 binds to the GC box element on target gene promoters to drive basal transcription **[A]**. Upon TGF-β stimulation, R-Smads and Smad4 form transcriptional complexes with Sp1 and induce gene transcription **[B]**. In cells that highly express DLX4, DLX4 forms an inactive complex in the nucleus by directly binding to both Sp1 and Smad4 and represses gene transcription **[C]**. TGF-β stimulation activates R-Smads and induces their translocation into the nucleus. However, activated R-Smads fail to bind to Smad4 due to the blocking effect of DLX4. As a consequence, TGF-β cannot induce transcription of target genes **[D]**.



2. Interaction of DLX4 and Sp1

Binding affinity and selectivity of Smad complexes for target gene promoters are principally dictated by interactions of Smads with other DNA-binding factors (10, 201). Similarly, binding affinity and selectivity of several homeodomain proteins are modulated by interactions with other transcriptional regulators (204, 205). Sp1 is an important binding partner of Smad proteins that cooperates with Smads to induce transcription of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$. Upon TGF-β stimulation, Sp1 binds to MH1 domain of Smad4 and the MH2 domain of Smad2 to form Sp1/Smad complexes that bind to the promoters via their respective binding sites (37, 51). Surprisingly, we observed that DLX4 does not prevent Sp1 from associating with Smad4 in TGF-β-stimulated cells (Figure 32A). Notably, DLX4 seemed to facilitate Smad4-Sp1 interactions even in the absence of TGF-β stimulation (Figure 32A). Furthermore, my studies demonstrate that DLX4 directly binds the DNA-binding domain of Sp1 and impairs the DNA-binding ability of Sp1 (Figures 34, 35 and 36). This study is the first to demonstrate that a homeodomain protein directly interacts with Sp1 and modulates Sp1 activity. Since my studies demonstrated that DLX4 directly binds to Smad4 and to Sp1, we speculate that DLX4 inhibits $p15^{Ink4B}$ transcription by two integrated mechanisms. Firstly, DLX4 might recruit Sp1 and Smad4 to form a transcriptionally inactive DLX4-Smad-Sp1 complex that is unable to bind TGF-β-activated R-Smads. Secondly, by directly binding to the DNA-binding domains of Smad4 and of Sp1, DLX4 might dislodge Smad4 and Sp1 from the $p15^{Ink4B}$ promoter (Figure 37). Because transcription of $p21^{WAF1/Cip1}$ is also induced by TGF-β via cooperative interactions

between Sp1 and Smad proteins (38), DLX4 might inhibit $p21^{WAF1/Cip1}$ transcription by a similar mechanism.

3. Induction of c-*myc* by DLX4

My studies indicate that DLX4 induces expression of c-myc by two mechanisms. On one hand, DLX4 can block the ability of TGF-β to repress c-*myc* promoter activity (Figure 20). As previously discussed in Chapter 1, interaction of the repressive complex comprising Smad3, Smad4 and E2F4/5, DP1 and p107 with the TIE element in the c-*myc* promoter blocks c-*myc* transcription (46, 47) (Figure 5A). Because DLX4 blocks Smad4 from binding activated Smad3 (Figure 27A), DLX4 might block formation of the repressive complex and thereby derepress the c-*myc* promoter. My studies also demonstrate that DLX4 induces c-myc expression independently of TGF-β/Smad signaling. One possibility is that DLX4 directly activates the c-*myc* promoter. The c-*myc* promoter contains multiple TAAT core motifs that are recognized by homeodomain proteins. Indeed, it has been reported that another homeodomain protein, HOXB4, induces c-*myc* promoter activity (206).

Our finding that DLX4 induces expression of c-myc independently of TGF-β signaling has several implications. Firstly, induction of c-myc by DLX4 provides a competing mitogenic signal against the TGF-β cytostatic program. c-myc induces expression of numerous cell cycle facilitators such as Id2, cdc25A and cyclin D1 (57, 58, 207, 208). Secondly, DLX4-induced c-myc expression might lead to down-regulated expression of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, because transcription of these genes

is repressed by c-myc (62, 63). The repression of $p15^{Ink4B}$ transcription by c-*myc* has some similarity to our observations with DLX4. We found that interaction of Sp1 with Smad4 was not disrupted by DLX4. Feng *et al.*, likewise, reported that c-myc does not compete with Sp1 for interaction with Smads (62). These authors speculated that by interacting with activated Smad2/3, c-myc promotes the formation of an inactive transcription complex with Smad proteins and Sp1. However, there are notable differences in the mechanisms by which DLX4 and c-myc repress $p15^{Ink4B}$ promoter activity. In my studies, DLX4 was found to inhibit interactions between Smad4 and R-Smads, and to inhibit R-Smad transcriptional activity. In contrast, c-myc does not inhibit interactions between Smad4 and R-Smads (62). Furthermore, in my studies, DLX4 was found to inhibit DNA-binding activity of Sp1. In contrast, c-myc does not affect binding of Sp1 to the $p15^{Ink4B}$ promoter (62). DLX4 might, therefore, repress $p15^{Ink4B}$ and possibly also $p21^{WAF1/Cip1}$ transcription by three distinct, but integrated, mechanisms: i) by increasing c-myc expression, ii) by preventing Smad4 from binding R-Smads, and iii) by blocking Sp1 DNA-binding activity.

B. INTERACTIONS BETWEEN *DLX* GENES AND THE TGF SUPER-FAMILY

1. Cross-regulation of *DLX* genes and TGF super-family members

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 (1, 129). 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, we found that DLX4 inhibited induction of transcriptional activity by BMP-4 (Figure 26). DLX1 has been reported to inhibit activin signaling (184). Conversely, signaling by TGF super-family members can modulate expression or activity of DLX proteins. For example, BMP-2 activates *DLX3* transcription (182). Smad6, an antagonist of BMP signaling, inhibits DLX3 transcriptional activity (183). Interestingly, we observed that levels of DLX4 protein decreased in cells following TGF-β stimulation (Figure 18). 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.

2. Binding specificity of homeodomain proteins to Smads

Transcription factors encoded by homeobox genes are characterized by their conserved helix-turn-helix DNA-binding homeodomain (209). Our findings that binding of DLX4 to the MH1 domain of Smad4 is mediated in part through the homeodomain of DLX4 raises the question of specificity. Transcription factors encoded by various homeobox genes have been reported to bind Smads, but not all of these interactions are solely mediated via the homeodomain. For example, proteins encoded by the *Mixer* and *Milk* homeobox genes bind Smad2 through a region distinct from their homeodomains (181). DLX1 has been reported to bind Smad4, but its binding region has not been identified (184). Transcription factors encoded by various other homeobox genes have been reported to bind Smads via their homeodomain. However, the specificity of homeodomain proteins for different Smads is striking. Most

of the interactions between homeodomain proteins and Smads reported so far are with either R-Smads or I-Smads. DLX3 binds Smad6 but not Smad4 (183). HOXA13 binds Smad1, Smad2 and Smad5 but does not bind Smad4 (185). Furthermore, different homeodomain proteins bind different domains of Smad proteins. For example, HOXC8 interacts with the MH1 domain of Smad1 (210), whereas HOXA13 binds the MH2 domains of Smad1 (185). Within the homeodomain, the residues of the third helix are the most highly conserved (127). Less conserved residues of the first and second helices might govern preferential binding to a specific Smad protein or Smad domain. My study is the first to demonstrate a direct interaction of a homeodomain protein to the MH1 domain of Smad4. Indeed, very few transcription factors are known to bind the MH1 domain of Smad4 (reviewed in (203)). However, it is possible that other homeodomain proteins could bind Smad4 and potentially block TGF-β-mediated growth inhibition by a mechanism similar to that of DLX4. Nonetheless, the specificity of this inhibition could largely depend on the context of the expression of homeobox genes. Most homeobox genes are expressed in a highly tissue-specific manner (137, 209). In contrast, DLX4 is expressed across diverse malignancies (Table 5). No other homeobox gene has been reported to be commonly expressed in tumors of lung, breast, ovary, prostate and hematologic origin. Interference of TGF-β-mediated growth inhibition by DLX4 could, therefore, be a mechanism common to multiple organ sites.

C. THE ROLE OF HOMEOBOX GENE *DLX4* IN TUMORIGENESIS

1. Resistance of tumors to the anti-proliferative effect of TGF-β

Resistance to TGF-β-mediated growth-inhibition is an important feature in the pathogenesis of most types of tumors (3, 4, 95). This resistance has been attributed to TGF-β receptor or Smad mutations in several types of tumors, particularly those of gastrointestinal and pancreatic origin (Table 2). Resistance of tumor cells to the anti-proliferative effect of TGF-β can also stem from down-regulation of TGF-β receptor expression (211), activation of Smad repressors, repression of Smad activators (Figure 2) and mutation of downstream targets such as $p15^{Ink4B}$ deletion (212). The ability of DLX4 to block TGF-β-mediated growth inhibition could explain why tumors that lack aberrations in core components of the TGF-β signaling pathway can become resistant to the anti-proliferative effect of TGF-β. It would be important in future studies to determine whether DLX4 is aberrantly activated in tumors that have TGF-β receptor or Smad mutations, and what is the effect of DLX4 in these tumors. It is interesting to note that Ski and SnoN proteins also can block the anti-proliferative effect of TGF-β and that these proteins are both elevated in many types of cancer. The *sno* gene is also located in a chromosomal locus that is frequently amplified in many tumors (reviewed in (29)). However, the mechanisms for the blocking effect of Ski/Sno are distinct from that of DLX4. Ski/Sno blocks TGF-β/Smad signaling in a "gene non-specific" manner by recruiting N-CoR/mSin3/HDAC repressor complexes to Smad4/R-Smad transcription complexes (27-29). However, in this study, we demonstrated that DLX4 blocks TGF-β/Smad signaling by both "gene non-specific" and "gene specific"

mechanisms. As discussed earlier, DLX4 blocks TGF-β/Smad signaling by a "gene non-specific" mechanism via binding Smad4 and preventing binding of Smad4 to TGF-β-activated R-Smads. DLX4 also blocks TGF-β-mediated induction of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ transcription by a "gene specific" mechanism via forming a transcriptionally inactive complex with Smad4 and Sp1, and by inducing expression of c-myc, a repressor of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$ transcription.

2. Other roles of DLX4 in tumor progression

A striking aspect of the TGF-β signaling pathway in tumors is its biphasic function. Many tumors are resistant to the anti-proliferative effect of TGF-β but retain other TGF-β-mediated mechanisms that promote EMT and metastasis (3, 4). 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 (3, 4). 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 (Figure 21). 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.* (178). However, DLX4 levels in ovarian and breast cancers have been reported to correlate with disease progression (173, 176). There are several possible explanations for this paradox. As described in Chapter 1, TGF-β not only induces EMT by Smad-dependent mechanisms, but also via Smad-independent pathways that involve MAP kinase and

RhoA activation (88). TGF-β-induced, non-Smad pathways that promote cell migration might not be inhibited by DLX4. One way to test this possibility is to assay the effect of DLX4 on cell migration using MDA-MB-468 breast cancer cells that have homozygous deletion of Smad4. DLX4 could also promote tumor progression by other mechanisms such as sustained induction of c-myc. For example, c-myc not only stimulates cell growth but also promotes tumor metastasis (213). In addition, our laboratory has found that DLX4 promotes tumor angiogenesis by inducing expression of vascular endothelial growth factor and fibroblast growth factor-2 (FGF-2) (173). 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 (175, 214). However, DLX4 overexpression occurs in >50% of these tumors (173, 176) indicating that gene amplification is not the sole mechanism underlying this overexpression.

3. Functional significance of homeobox genes in cell growth deregulation in cancers

In addition to the ability of DLX4 to block the TGF-β anti-growth signal identified in this study, other reports implicate a function for DLX4 in enhancing cell survival. The anti-apoptotic effect of DLX4 has been associated with its ability to induce expression of GATA-1 and bcl-2 (171, 190). DLX4 has also been reported to repress expression of the DNA-damage repair protein BRCA1 in breast cancer, suggesting that DLX4 might play a role in DNA-damage repair (189). Aberrant expression of other homeobox genes can also promote tumor cell survival and growth. *HOXA1* is overexpressed in

breast cancers and promotes tumor cell survival by inducing bcl-2 expression (215). *HSIX1* is also highly expressed in breast cancers and causes abrogation of the G_2 checkpoint by inducing expression of cyclin A1 (162, 163). *HOXB7* is overexpressed in melanomas, breast and ovarian cancers and induces expression of FGF-2 (167, 216). On the other hand, several homeobox genes can inhibit cell growth, and their expression is lost in tumors. *CDX2* inhibits cell growth by inducing expression of $p21^{WAF1/Cip1}$, and *CDX2* is down-regulated in colon cancers (152, 217). *p53* is a direct transcriptional target of HOXA5 (145). Loss of *HOXA5* expression has been reported in >60% of breast cancers (145). My study 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. This study significantly supports a growing body of evidence that aberrant expression of homeobox genes can deregulate tumor cell growth by a wide variety of different mechanisms.

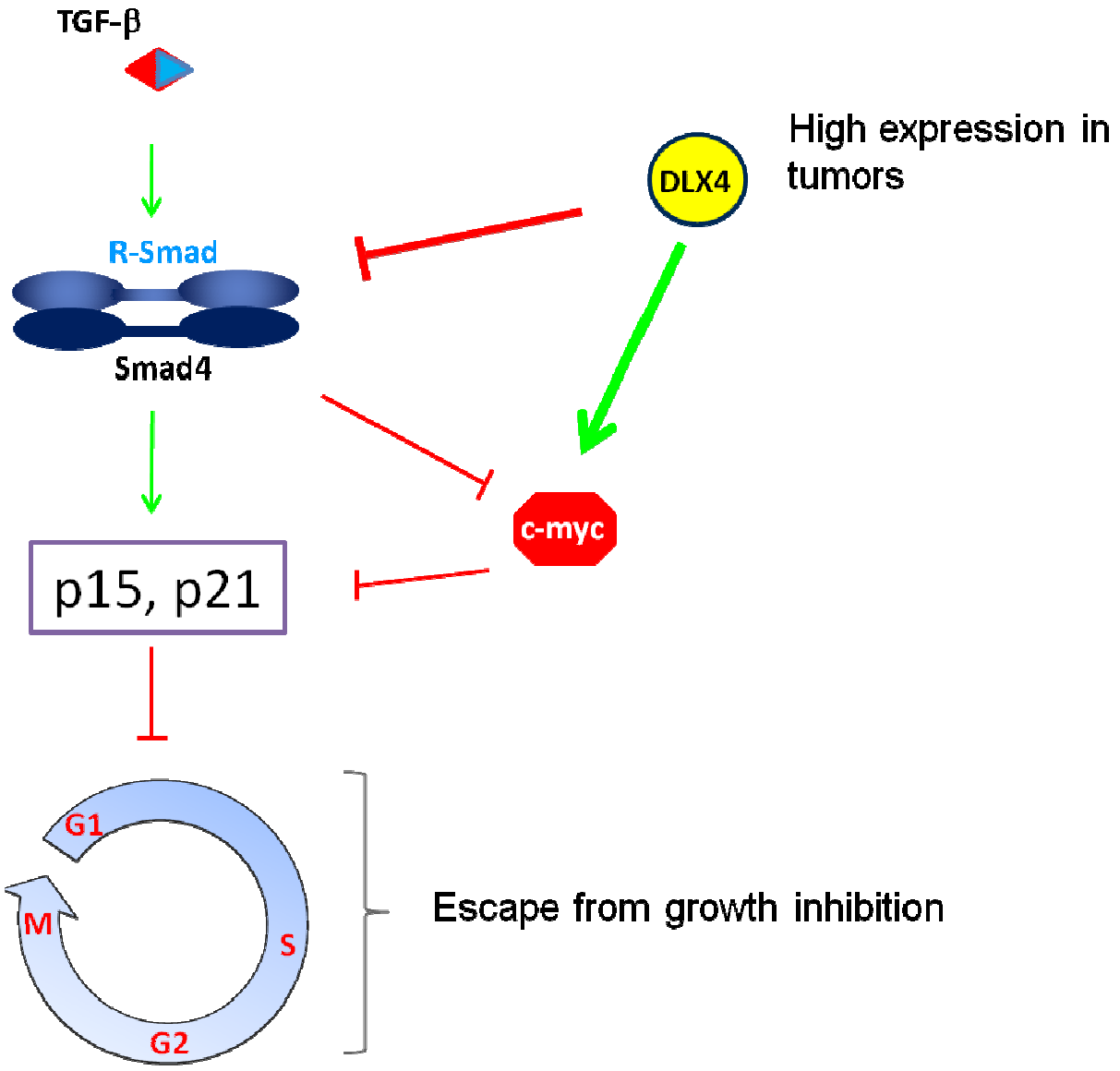
D. CONCLUSION

Resistance to the anti-proliferative effect of TGF-β is an important feature in the pathogenesis of most types of cancers. Resistance of many tumors to TGF-β cannot be solely explained by TGF-β receptor and Smad mutations or deletions. My studies demonstrate that DLX4 blocks the anti-proliferative effect of TGF-β by disabling key transcriptional control mechanisms of the TGF-β cytostatic program. My studies also provide a molecular explanation as to why many tumors are resistant to the growth-inhibitory effect of TGF-β in the absence of mutations in core components of the TGF-β signaling pathway (Figure 38). Escape from anti-proliferative signals is an important

and early step in tumor pathogenesis. DLX4 might therefore, serve as a useful early detection marker and therapeutic target for multiple types of tumors. At a broader level, my studies might provide insight into how aberrant activation of a developmental patterning gene promotes tumor pathogenesis. Conversely, the finding of this study that DLX4 modulates TGF-β signaling in tumors also provides insights into how homeobox genes and TGF-β signaling interact to control normal developmental patterning.

Figure 38. DLX4 blocks TGF-β-induced G_1 arrest

TGF-β induces G_1 arrest primarily by inducing expression of CDK inhibitors, $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$. DLX4 prevents G_1 arrest by blocking TGF-β/Smad signaling and by inducing expression of c-myc, a transcriptional repressor of $p15^{Ink4B}$ and $p21^{WAF1/Cip1}$, independently of TGF-β signaling.



BIBLIOGRAPHY

1. Wu, M. Y., and C. S. Hill. 2009. TGF-beta superfamily signaling in embryonic development and homeostasis. Dev Cell 16:329-343.
2. Thiery, J. P., H. Acloque, R. Y. J. Huang, and M. A. Nieto. 2009. Epithelial-mesenchymal transitions in development and disease. Cell 139:871-890.
3. Siegel, P. M., and J. Massague. 2003. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer 3:807-820.
4. Massague, J. 2008. TGF-beta in cancer. Cell 134:215-230.
5. Massague, J., and D. Wotton. 2000. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J 19:1745-1754.
6. Imamura, T., M. Takase, A. Nishihara, E. Oeda, J. Hanai, M. Kawabata, and K. Miyazono. 1997. Smad6 inhibits signalling by the TGF-beta superfamily. Nature 389:622-626.
7. Nakao, A., M. Afrakhte, A. Moren, T. Nakayama, J. L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N. E. Heldin, C. H. Heldin, and P. ten Dijke. 1997. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature 389:631-635.
8. Massague, J., J. Seoane, and D. Wotton. 2005. Smad transcription factors. Genes Dev 19:2783-2810.
9. Derynck, R., and Y. E. Zhang. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577-584.
10. Feng, X. H., and R. Derynck. 2005. Specificity and versatility in TGF-beta signaling through Smads. Annu Rev Cell Dev Biol 21:659-693.

11. Koli, K., J. Saharinen, M. Hyytiainen, C. Penttinen, and J. Keski-Oja. 2001. Latency, activation, and binding proteins of TGF-beta. Microsc Res Tech 52:354-362.

12. Massague, J. 1998. TGF-beta signal transduction. Annu Rev Biochem 67:753-791.

13. Bakin, A. V., A. K. Tomlinson, N. A. Bhowmick, H. L. Moses, and C. L. Arteaga. 2000. Phosphatidylinositol 3-Kinase function is required for transforming growth factor-beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem 275:36803-36810.

14. Ozdamar, B., R. Bose, M. Barrios-Rodiles, H.-R. Wang, Y. Zhang, and J. L. Wrana. 2005. Regulation of the polarity protein Par6 by TGF-beta receptors controls epithelial cell plasticity. Science 307:1603-1609.

15. Yu, L., M. C. Hebert, and Y. E. Zhang. 2002. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J 21:3749-3759.

16. Yi, J. Y., I. Shin, and C. L. Arteaga. 2005. Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. J Biol Chem 280:10870-10876.

17. Yilmaz, M., and G. Christofori. 2009. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 28:15-33.

18. Suzuki, C., G. Murakami, M. Fukuchi, T. Shimanuki, Y. Shikauchi, T. Imamura, and K. Miyazono. 2002. Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. J Biol Chem 277:39919-39925.

19. Kavsak, P., R. K. Rasmussen, C. G. Causing, S. Bonni, H. Zhu, G. H. Thomsen, and J. L. Wrana. 2000. Smad7 binds to Smurf2 to form an E3

ubiquitin ligase that targets the TGF-beta receptor for degradation. Mol Cell 6:1365-1375.

20. Hayashi, H., S. Abdollah, Y. Qiu, J. Cai, Y. Y. Xu, B. W. Grinnell, M. A. Richardson, J. N. Topper, M. A. Gimbrone, Jr., J. L. Wrana, and D. Falb. 1997. The MAD-related protein Smad7 associates with the TGF-beta receptor and functions as an antagonist of TGF-beta signaling. Cell 89:1165-1173.

21. Derynck, R., R. J. Akhurst, and A. Balmain. 2001. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet 29:117-129.

22. Inman, G. J., F. J. Nicolas, and C. S. Hill. 2002. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. Mol Cell 10:283-294.

23. ten Dijke, P., and C. S. Hill. 2004. New insights into TGF-beta-Smad signalling. Trends Biochem Sci 29:265-273.

24. Lin, X., X. Duan, Y. Y. Liang, Y. Su, K. H. Wrighton, J. Long, M. Hu, C. M. Davis, J. Wang, F. C. Brunicardi, Y. Shi, Y. G. Chen, A. Meng, and X. H. Feng. 2006. PPM1A functions as a Smad phosphatase to terminate TGF-beta signaling. Cell 125:915-928.

25. Kretzschmar, M., J. Doody, I. Timokhina, and J. Massague. 1999. A mechanism of repression of TGF-beta/Smad signaling by oncogenic Ras. Genes Dev 13:804-816.

26. Wotton, D., R. Lo, S. Lee, and J. Massague. 1999. A Smad transcriptional corepressor. Cell 97:29-39.

27. Akiyoshi, S., H. Inoue, J. Hanai, K. Kusanagi, N. Nemoto, K. Miyazono, and M. Kawabata. 1999. c-Ski acts as a transcriptional co-repressor in transforming

growth factor-beta signaling through interaction with smads. J Biol Chem 274:35269-35277.

28. Stroschein, S. L., W. Wang, S. Zhou, Q. Zhou, and K. Luo. 1999. Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. Science 286:771-774.

29. Luo, K. 2004. Ski and SnoN: negative regulators of TGF-beta signaling. Curr Opin Genet Dev 14:65-70.

30. Abdollah, S., M. Macias-Silva, T. Tsukazaki, H. Hayashi, L. Attisano, and J. L. Wrana. 1997. TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J Biol Chem 272:27678-27685.

31. Macias-Silva, M., S. Abdollah, P. A. Hoodless, R. Pirone, L. Attisano, and J. L. Wrana. 1996. MADR2 is a substrate of the TGF-beta receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell 87:1215-1224.

32. Brown, K. A., J. A. Pietenpol, and H. L. Moses. 2007. A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling. J Cell Biochem 101:9-33.

33. Shi, Y., Y. F. Wang, L. Jayaraman, H. Yang, J. Massague, and N. P. Pavletich. 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell 94:585-594.

34. Ross, S., E. Cheung, T. G. Petrakis, M. Howell, W. L. Kraus, and C. S. Hill. 2006. Smads orchestrate specific histone modifications and chromatin remodeling to activate transcription. EMBO J 25:4490-4502.

35. Attisano, L., and J. L. Wrana. 2000. Smads as transcriptional co-modulators. Curr Opin Cell Biol 12:235-243.

36. Li, J.-M., M. A. Nichols, S. Chandrasekharan, Y. Xiong, and X.-F. Wang. 1995. Transforming growth factor beta activates the promoter of Cyclin-dependent kinase inhibitor p15Ink4B through an Sp1 consensus site. J. Biol. Chem. 270:26750-26753.

37. Moustakas, A., and D. Kardassis. 1998. Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. Proc Natl Acad Sci U S A 95:6733-6738.

38. Pardali, K., A. Kurisaki, A. Moren, P. ten Dijke, D. Kardassis, and A. Moustakas. 2000. Role of Smad proteins and transcription factor Sp1 in p21WAF1/Cip1 regulation by transforming growth factor-beta. J Biol Chem 275:29244-29256.

39. Akhurst, R. J., and R. Derynck. 2001. TGF-beta signaling in cancer - a double-edged sword. Trends Cell Biol 11:S44-S51.

40. Bannister, A. J., and T. Kouzarides. 1996. The CBP co-activator is a histone acetyltransferase. Nature 384:641-643.

41. Feng, X. H., Y. Zhang, R. Y. Wu, and R. Derynck. 1998. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. Genes Dev 12:2153-2163.

42. Janknecht, R., N. J. Wells, and T. Hunter. 1998. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev 12:2114-2119.

43. Itoh, S., J. Ericsson, J. Nishikawa, C. H. Heldin, and P. ten Dijke. 2000. The transcriptional co-activator P/CAF potentiates TGF-beta/Smad signaling. Nucleic Acids Res 28:4291-4298.

44. Rushlow, C., P. F. Colosimo, M. Lin, M. Xu, and N. Kirov. 2001. Transcriptional regulation of the Drosophila gene zen by competing Smad and Brinker inputs. Genes Dev 15:340-351.

45. Zavadil, J., M. Bitzer, D. Liang, Y. C. Yang, A. Massimi, S. Kneitz, E. Piek, and E. P. Bottinger. 2001. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. Proc Natl Acad Sci U S A 98:6686-6691.

46. Chen, C. R., Y. Kang, P. M. Siegel, and J. Massague. 2002. E2F4/5 and p107 as Smad cofactors linking the TGF-beta receptor to c-myc repression. Cell 110:19-32.

47. Frederick, J. P., N. T. Liberati, D. S. Waddell, Y. Shi, and X. F. Wang. 2004. Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. Mol Cell Biol 24:2546-2559.

48. Kang, Y., C.-R. Chen, and J. Massague. 2003. A self-enabling TGF-beta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol Cell 11:915-926.

49. Kang, J. S., T. Alliston, R. Delston, and R. Derynck. 2005. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. EMBO J 24:2543-2555.

50. Liu, D., B. L. Black, and R. Derynck. 2001. TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. Genes Dev 15:2950-2966.

51. Feng, X.-H., X. Lin, and R. Derynck. 2000. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15Ink4B transcription in response to TGF-beta. EMBO J 19:5178-5193.

52. Mulder, K., A. Levine, X. Hernandez, M. McKnight, D. Brattain, and M. Brattain. 1988. Modulation of c-myc by transforming growth factor-beta in human colon carcinoma cells. Biochem Biophys Res Commun 150:711-716.

53. Hannon, G. J., and D. Beach. 1994. p15Ink4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature 371:257-261.

54. Wade Harper, J., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805-816.

55. Chen, H. Z., S. Y. Tsai, and G. Leone. 2009. Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer 9:785-797.

56. Harbour, J. W., R. X. Luo, A. Dei Santi, A. A. Postigo, and D. C. Dean. 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98:859-869.

57. Galaktionov, K., X. Chen, and D. Beach. 1996. Cdc25 cell-cycle phosphatase as a target of c-myc. Nature 382:511-517.

58. Lasorella, A., M. Noseda, M. Beyna, Y. Yokota, and A. Iavarone. 2000. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature 407:592-598.

59. Zebedee, Z., and E. Hara. 2001. Id proteins in cell cycle control and cellular senescence. Oncogene 20:8317-8325.

60. Petritsch, C., H. Beug, A. Balmain, and M. Oft. 2000. TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G1 arrest. Genes Dev 14:3093-3101.

61. Lane, H. A., A. Fernandez, N. J. C. Lamb, and G. Thomas. 1993. p70s6k function is essential for G1 progression. Nature 363:170-172.

62. Feng, X.-H., Y.-Y. Liang, M. Liang, W. Zhai, and X. Lin. 2002. Direct Interaction of c-Myc with Smad2 and Smad3 to Inhibit TGF-beta-mediated Induction of the CDK Inhibitor p15Ink4B. Mol Cell 9:133-143.

63. Gartel, A. L., X. Ye, E. Goufman, P. Shianov, N. Hay, F. Najmabadi, and A. L. Tyner. 2001. Myc represses the p21WAF1/Cip1 promoter and interacts with Sp1/Sp3. Proc Natl Acad Sci U S A 98:4510-4515.

64. Siegel, P. M., W. Shu, and J. Massague. 2003. Mad upregulation and Id2 repression accompany transforming growth factor-beta-mediated epithelial cell growth suppression. J Biol Chem 278:35444-35450.

65. Huang, Y., D. Hutter, Y. Liu, X. Wang, M. S. Sheikh, A. M.-L. Chan, and N. J. Holbrook. 2000. Transforming growth factor-beta suppresses serum deprivation-induced death of A549 cells through differential effects on c-Jun and JNK activities. J Biol Chem 275:18234-18242.

66. Jang, C.-W., C.-H. Chen, C.-C. Chen, J.-y. Chen, Y.-H. Su, and R.-H. Chen. 2002. TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. Nat Cell Biol 4:51-58.

67. Shin, I., A. V. Bakin, U. Rodeck, A. Brunet, and C. L. Arteaga. 2001. Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. Mol. Biol. Cell 12:3328-3339.

68. Tachibana, I., M. Imoto, P. N. Adjei, G. J. Gores, M. Subramaniam, T. C. Spelsberg, and R. Urrutia. 1997. Overexpression of the TGF-beta-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. J Clin Invest 99:2365-2374.

69. Valderrama-Carvajal, H., E. Cocolakis, A. Lacerte, E.-H. Lee, G. Krystal, S. Ali, and J.-J. Lebrun. 2002. Activin/TGF-beta induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP. Nat Cell Biol 4:963-969.

70. De Craene, B., B. Gilbert, C. Stove, E. Bruyneel, F. van Roy, and G. Berx. 2005. The transcription factor Snail induces tumor cell Invasion through modulation of the epithelial cell differentiation program. Cancer Res 65:6237-6244.

71. Cano, A., M. A. Perez-Moreno, I. Rodrigo, A. Locascio, M. J. Blanco, M. G. del Barrio, F. Portillo, and M. A. Nieto. 2000. The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2:76-83.

72. Ikenouchi, J., M. Matsuda, M. Furuse, and S. Tsukita. 2003. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. J Cell Sci 116:1959-1967.

73. Moreno-Bueno, G., E. Cubillo, D. Sarrio, H. Peinado, S. M. Rodriguez-Pinilla, S. Villa, V. Bolos, M. Jorda, A. Fabra, F. Portillo, J. Palacios, and A. Cano. 2006. Genetic profiling of epithelial cells expressing E-cadherin repressors reveals a distinct role for Snail, Slug, and E47 factors in epithelial-mesenchymal transition. Cancer Res 66:9543-9556.

74. Olmeda, D., M. Jorda, H. Peinado, A. Fabra, and A. Cano. 2006. Snail silencing effectively suppresses tumour growth and invasiveness. Oncogene 26:1862-1874.

75. Barrallo-Gimeno, A., and M. A. Nieto. 2005. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 132:3151-3161.

76. Comijn, J., G. Berx, P. Vermassen, K. Verschueren, L. van Grunsven, E. Bruyneel, M. Mareel, D. Huylebroeck, and F. van Roy. 2001. The Two-handed E box binding Zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell 7:1267-1278.

77. Eger, A., K. Aigner, S. Sonderegger, B. Dampier, S. Oehler, M. Schreiber, G. Berx, A. Cano, H. Beug, and R. Foisner. 2005. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene 24:2375-2385.

78. Vandewalle, C., J. Comijn, B. De Craene, P. Vermassen, E. Bruyneel, H. Andersen, E. Tulchinsky, F. Van Roy, and G. Berx. 2005. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. Nucleic Acids Res 33:6566-6578.

79. Yang, J., S. A. Mani, J. L. Donaher, S. Ramaswamy, R. A. Itzykson, C. Come, P. Savagner, I. Gitelman, A. Richardson, and R. A. Weinberg. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117:927-939.

80. Bakin, A. V., C. Rinehart, A. K. Tomlinson, and C. L. Arteaga. 2002. p38 mitogen-activated protein kinase is required for TGF-beta-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci 115:3193-3206.

81. Xie, L., B. K. Law, A. M. Chytil, K. A. Brown, M. E. Aakre, and H. L. Moses. 2004. Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. Neoplasia 6:603-610.

82. Alcorn, J. F., A. S. Guala, J. van der Velden, B. McElhinney, C. G. Irvin, R. J. Davis, and Y. M. Janssen-Heininger. 2008. Jun N-terminal kinase 1 regulates

epithelial-to-mesenchymal transition induced by TGF-beta1. J Cell Sci 121:1036-1045.

83. Tavares, A. L., M. E. Mercado-Pimentel, R. B. Runyan, and G. T. Kitten. 2006. TGF-beta-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart. Dev Dyn 235:1589-1598.

84. Sorrentino, A., N. Thakur, S. Grimsby, A. Marcusson, V. von Bulow, N. Schuster, S. Zhang, C. H. Heldin, and M. Landstrom. 2008. The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol 10:1199-1207.

85. Yamashita, M., K. Fatyol, C. Jin, X. Wang, Z. Liu, and Y. E. Zhang. 2008. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. Mol Cell 31:918-924.

86. Lee, M. K., C. Pardoux, M. C. Hall, P. S. Lee, D. Warburton, J. Qing, S. M. Smith, and R. Derynck. 2007. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. EMBO J 26:3957-3967.

87. Willis, B. C., and Z. Borok. 2007. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 293:L525-534.

88. Moustakas, A., and C. H. Heldin. 2005. Non-Smad TGF-beta signals. J Cell Sci 118:3573-3584.

89. Edlund, S., M. Landström, C. H. Heldin, and P. Aspenström. 2002. Transforming growth factor-beta–induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. Mol Biol Cell 13:902-914.

90. Ishizaki, T., M. Naito, K. Fujisawa, M. Maekawa, N. Watanabe, Y. Saito, and S. Narumiya. 1997. p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Letts 404:118-124.

91. Zhu, B., K. Fukada, H. Zhu, and N. Kyprianou. 2006. Prohibitin and cofilin are intracellular effectors of transforming growth factor-beta signaling in human prostate cancer cells. Cancer Res 66:8640-8647.

92. Larue, L., and A. Bellacosa. 2005. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 24:7443-7454.

93. Grille, S. J., A. Bellacosa, J. Upson, A. J. Klein-Szanto, F. van Roy, W. Lee-Kwon, M. Donowitz, P. N. Tsichlis, and L. Larue. 2003. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res 63:2172-2178.

94. Kim, D., S. Kim, H. Koh, S. O. Yoon, A. S. Chung, K. S. Cho, and J. Chung. 2001. Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. FASEB J 15:1953-1962.

95. Hanahan, D., and R. Weinberg. 2000. The hallmarks of cancer. Cell 100:57-70.

96. Oft, M., K. H. Heider, and H. Beug. 1998. TGF-beta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol 8:1243-1252.

97. Ueki, N., M. Nakazato, T. Ohkawa, T. Ikeda, Y. Amuro, T. Hada, and K. Higashino. 1992. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. Biochim Biophys Acta 1137:189-196.

98. Madri, J. A., B. M. Pratt, and A. M. Tucker. 1988. Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. J Cell Biol 106:1375-1384.

99. Stearns, M. E., F. U. Garcia, K. Fudge, J. Rhim, and M. Wang. 1999. Role of interleukin 10 and transforming growth factor beta1 in the angiogenesis and metastasis of human prostate primary tumor lines from orthotopic implants in severe combined immunodeficiency mice. Clin Cancer Res 5:711-720.

100. Saito, H., S. Tsujitani, S. Oka, A. Kondo, M. Ikeguchi, M. Maeta, and N. Kaibara. 1999. The expression of transforming growth factor-beta1 is significantly correlated with the expression of vascular endothelial growth factor and poor prognosis of patients with advanced gastric carcinoma. Cancer 86:1455-1462.

101. Botti, C., E. Seregni, L. Ferrari, A. Martinetti, and E. Bombardieri. 1998. Immunosuppressive factors: role in cancer development and progression. Int J Biol Markers 13:51-69.

102. Letterio, J. J., and A. B. Roberts. 1998. Regulation of immune responses by TGF-beta. Annu Rev Immunol 16:137-161.

103. Torre-Amione, G., R. D. Beauchamp, H. Koeppen, B. H. Park, H. Schreiber, H. L. Moses, and D. A. Rowley. 1990. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. Proc Natl Acad Sci U S A 87:1486-1490.

104. Inge, T. H., S. K. Hoover, B. M. Susskind, S. K. Barrett, and H. D. Bear. 1992. Inhibition of tumor-specific cytotoxic T-lymphocyte responses by transforming growth factor beta 1. Cancer Res 52:1386-1392.

105. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 163:1037-1050.

106. Letterio, J. J., A. G. Geiser, A. B. Kulkarni, H. Dang, L. Kong, T. Nakabayashi, C. L. Mackall, R. E. Gress, and A. B. Roberts. 1996. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. J Clin Invest 98:2109-2119.

107. Geiser, A. G., J. J. Letterio, A. B. Kulkarni, S. Karlsson, A. B. Roberts, and M. B. Sporn. 1993. Transforming growth factor beta 1 (TGF-beta 1) controls expression of major histocompatibility genes in the postnatal mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-beta 1 null mouse phenotype. Proc Natl Acad Sci U S A 90:9944-9948.

108. Czarniecki, C. W., H. H. Chiu, G. H. Wong, S. M. McCabe, and M. A. Palladino. 1988. Transforming growth factor-beta 1 modulates the expression of class II histocompatibility antigens on human cells. J Immunol 140:4217-4223.

109. Horwitz, D. A., J. D. Gray, K. Ohtsuka, M. Hirokawa, and T. Takahashi. 1997. The immunoregulatory effects of NK cells: the role of TGF-beta and implications for autoimmunity. Immunol Today 18:538-542.

110. Chen, J. J., Y. Sun, and G. J. Nabel. 1998. Regulation of the proinflammatory effects of Fas ligand (CD95L). Science 282:1714-1717.

111. Kehrl, J. H., A. B. Roberts, L. M. Wakefield, S. Jakowlew, M. B. Sporn, and A. S. Fauci. 1986. Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. J Immunol 137:3855-3860.

112. Levy L, and H. CS. 2006. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev 17:41-58.

113. Markowitz, S., J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler, B. Vogelstein, and et al. 1995. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268:1336-1338.

114. Grady, W. M., L. L. Myeroff, S. E. Swinler, A. Rajput, S. Thiagalingam, J. D. Lutterbaugh, A. Neumann, M. G. Brattain, J. Chang, S.-J. Kim, K. W. Kinzler, B. Vogelstein, J. K. V. Willson, and S. Markowitz. 1999. Mutational inactivation of transforming growth factor-beta receptor type II in microsatellite stable colon cancers. Cancer Res 59:320-324.

115. Woodford-Richens, K. L., A. J. Rowan, P. Gorman, S. Halford, D. C. Bicknell, H. S. Wasan, R. R. Roylance, W. F. Bodmer, and I. P. Tomlinson. 2001. SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. Proc Natl Acad Sci U S A 98:9719-9723.

116. Wang, D., T. Kanuma, H. Mizunuma, F. Takama, Y. Ibuki, N. Wake, A. Mogi, Y. Shitara, and S. Takenoshita. 2000. Analysis of specific gene mutations in the transforming growth factor-beta signal transduction pathway in human ovarian cancer. Cancer Res 60:4507-4512.

117. Francis-Thickpenny, K. M., D. M. Richardson, C. Ee, D. R. Love, I. M. Winship, B. C. Baguley, G. Chenevix-Trench, and A. N. Shelling. 2001. Analysis of the TGF-beta functional pathway in epithelial ovarian carcinoma. Br J Cancer 85:687-691.

118. Yamada, S. D., R. L. Baldwin, and B. Y. Karlan. 1999. Ovarian carcinoma cell cultures are resistant to TGF-beta1-mediated growth inhibition despite expression of functional receptors. Gynecol Oncol 75:72-77.

119. Nagatake, M., Y. Takagi, H. Osada, K. Uchida, T. Mitsudomi, S. Saji, K. Shimokata, and T. Takahashi. 1996. Somatic in vivo alterations of the DPC4 gene at 18q21 in human lung cancers. Cancer Res 56:2718-2720.

120. Bello-DeOcampo, D., and D. J. Tindall. 2003. TGF-betal/Smad signaling in prostate cancer. Curr Drug Targets 4:197-207.

121. Takenoshita, S., K. Hagiwara, A. Gemma, M. Nagashima, D. Ryberg, B. A. Lindstedt, W. P. Bennett, A. Haugen, and C. C. Harris. 1997. Absence of mutations in the transforming growth factor-beta type II receptor in sporadic lung cancers with microsatellite instability and rare H-ras1 alleles. Carcinogenesis 18:1427-1429.

122. Schutte, M., R. H. Hruban, L. Hedrick, K. R. Cho, G. M. Nadasdy, C. L. Weinstein, G. S. Bova, W. B. Isaacs, P. Cairns, H. Nawroz, D. Sidransky, R. A. Casero, Jr., P. S. Meltzer, S. A. Hahn, and S. E. Kern. 1996. DPC4 gene in various tumor types. Cancer Res 56:2527-2530.

123. Takenoshita, S., A. Mogi, M. Tani, H. Osawa, H. Sunaga, H. Kakegawa, Y. Yanagita, T. Koida, M. Kimura, K. I. Fujita, H. Kato, R. Kato, and Y. Nagamachi. 1998. Absence of mutations in the analysis of coding sequences of the entire transforming growth factor-beta type II receptor gene in sporadic human breast cancers. Oncol Rep 5:367-371.

124. Watanabe, T., T. T. Wu, P. J. Catalano, T. Ueki, R. Satriano, D. G. Haller, A. B. Benson, 3rd, and S. R. Hamilton. 2001. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. N Engl J Med 344:1196-1206.

125. Hahn, S. A., M. Schutte, A. T. Hoque, C. A. Moskaluk, L. T. da Costa, E. Rozenblum, C. L. Weinstein, A. Fischer, C. J. Yeo, R. H. Hruban, and S. E. Kern. 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271:350-353.

126. Tupler, R., G. Perini, and M. R. Green. 2001. Expressing the human genome. Nature 409:832-833.

127. Gehring, W. J., and Y. Hiromi. 1986. Homeotic genes and the homeobox. Annu Rev Genet 20:147-173.

128. Krumlauf, R. 1994. Hox genes in vertebrate development. Cell 78:191-201.

129. Panganiban, G., and J. L. R. Rubenstein. 2002. Developmental functions of the Distal-less/Dlx homeobox genes. Development 129:4371-4386.

130. Qiu, M., A. Bulfone, I. Ghattas, J. J. Meneses, L. Christensen, P. T. Sharpe, R. Presley, R. A. Pedersen, and J. L. Rubenstein. 1997. Role of the Dlx homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx-1, Dlx-2, and Dlx-1 and -2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. Dev Biol 185:165-184.

131. Abate-Shen, C. 2002. Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer 2:777-785.

132. Stein, S., R. Fritsch, L. Lemaire, and M. Kessel. 1996. Checklist: vertebrate homeobox genes. Mech Dev 55:91-108.

133. Zhong, Y. F., T. Butts, and P. W. H. Holland. 2008. HomeoDB: a database of homeobox gene diversity. Evol Dev 10:516-518.

134. Sumiyama, K., S. Irvine, and F. Ruddle. 2003. The role of gene duplication in the evolution and function of the vertebrate Dlx/distal-less bigene clusters. J Struct Funct Genomics 3:151-159.

135. Stock, D., D. Ellies, Z. Zhao, M. Ekker, F. Ruddle, and K. Weiss. 1996. The evolution of the vertebrate Dlx gene family. Proc Natl Acad Sci U S A 93:10858-10863.

136. Ferrier, D. E., and C. Minguillon. 2003. Evolution of the Hox/ParaHox gene clusters. Int J Dev Biol 47:605-611.

137. Zakany, J., M. Kmita, P. Alarcon, J. L. de la Pompa, and D. Duboule. 2001. Localized and transient transcription of Hox genes suggests a link between patterning and the segmentation clock. Cell 106:207-217.

138. Mark, M., F. M. Rijli, and P. Chambon. 1997. Homeobox genes in embryogenesis and pathogenesis. Pediatr Res 42:421-429.

139. Gorski, D., and K. Walsh. 2000. The role of homeobox genes in vascular remodeling and angiogenesis. Circ Res 87:865-872.

140. MacLean, J. n., and M. Wilkinson. 2010. The Rhox genes. Reproduction 140:195-213.

141. Sauvageau, G., U. Thorsteinsdottir, C. Eaves, H. Lawrence, C. Largman, P. Lansdorp, and R. Humphries. 1995. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev 9:1753-1765.

142. Owens, B., and R. Hawley. 2002. HOX and non-HOX homeobox genes in leukemic hematopoiesis. Stem Cells 20:364-379.

143. Taylor, H. S. 2000. The role of HOX genes in human implantation. Hum Reprod Update 6:75-79.

144. Samuel, S., and H. Naora. 2005. Homeobox gene expression in cancer: insights from developmental regulation and deregulation. Eur J Cancer 41:2428-2437.

145. Raman, V., S. Martensen, D. Reisman, E. Evron, W. Odenwald, E. Jaffee, J. Marks, and S. Sukumar. 2000. Compromised HOXA5 function can limit p53 expression in human breast tumors. Nature 405:974 - 978.

146. He, W. W., P. J. Sciavolino, J. Wing, M. Augustus, P. Hudson, P. S. Meissner, R. T. Curtis, B. K. Shell, D. G. Bostwick, D. J. Tindall, E. P. Gelmann, C. Abate-Shen, and K. C. Carter. 1997. A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 43:69-77.

147. Zeng, W. R., S. W. Scherer, M. Koutsilieris, J. J. Huizenga, F. Filteau, L. C. Tsui, and A. Nepveu. 1997. Loss of heterozygosity and reduced expression of the CUTL1 gene in uterine leiomyomas. Oncogene 14:2355-2365.

148. Nakamura, T., D. A. Largaespada, M. P. Lee, L. A. Johnson, K. Ohyashiki, K. Toyama, S. J. Chen, C. L. Willman, I. M. Chen, A. P. Feinberg, N. A. Jenkins, N. G. Copeland, and J. D. Shaughnessy, Jr. 1996. Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. Nat Genet 12:154-158.

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

150. Chawengsaksophak, K., R. James, V. E. Hammond, F. Kontgen, and F. Beck. 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. Nature 386:84-87.

151. Mallo, G. V., P. Soubeyran, J. C. Lissitzky, F. Andre, C. Farnarier, J. Marvaldi, J. C. Dagorn, and J. L. Iovanna. 1998. Expression of the Cdx1 and Cdx2 homeotic genes leads to reduced malignancy in colon cancer-derived cells. J Biol Chem 273:14030-14036.

152. Bai, Y. Q., S. Miyake, T. Iwai, and Y. Yuasa. 2003. CDX2, a homeobox transcription factor, upregulates transcription of the p21/WAF1/Cip1 gene. Oncogene 22:7942-7949.

153. Bhatia-Gaur, R., A. A. Donjacour, P. J. Sciavolino, M. Kim, N. Desai, P. Young, C. R. Norton, T. Gridley, R. D. Cardiff, G. R. Cunha, C. Abate-Shen, and M. M. Shen. 1999. Roles for Nkx3.1 in prostate development and cancer. Genes Dev 13:966-977.

154. Bowen, C., L. Bubendorf, H. J. Voeller, R. Slack, N. Willi, G. Sauter, T. C. Gasser, P. Koivisto, E. E. Lack, J. Kononen, O. P. Kallioniemi, and E. P. Gelmann. 2000. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res 60:6111-6115.

155. Henderson, G. S., P. J. van Diest, H. Burger, J. Russo, and V. Raman. 2006. Expression pattern of a homeotic gene, HOXA5, in normal breast and in breast tumors. Cell Oncol 28:305-313.

156. Sellar, G. C., L. Li, K. P. Watt, B. D. Nelkin, G. J. Rabiasz, E. A. Stronach, E. P. Miller, D. J. Porteous, J. F. Smyth, and H. Gabra. 2001. BARX2 induces cadherin 6 expression and is a functional suppressor of ovarian cancer progression. Cancer Res 61:6977-6981.

157. Sellar, G. C., K. P. Watt, L. Li, B. D. Nelkin, G. J. Rabiasz, D. J. Porteous, J. F. Smyth, and H. Gabra. 2002. The homeobox gene BARX2 can modulate cisplatin sensitivity in human epithelial ovarian cancer. Int J Oncol 21:929-933.

158. Daniel, L., E. Lechevallier, R. Giorgi, H. Sichez, H. Zattara-Cannoni, D. Figarella-Branger, and C. Coulange. 2001. Pax-2 expression in adult renal tumors. Human Pathol 32:282-287.

159. Dressler, G., U. Deutsch, K. Chowdhury, H. Nornes, and P. Gruss. 1990. Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. Development 109:787-795.

160. Muratovska, A., C. Zhou, S. He, P. Goodyer, and M. R. Eccles. 2003. Paired-box genes are frequently expressed in cancer and often required for cancer cell survival. Oncogene 22:7989-7997.

161. Ostrom, L., M. J. Tang, P. Gruss, and G. R. Dressler. 2000. Reduced Pax2 gene dosage increases apoptosis and slows the progression of renal cystic disease. Dev Biol 219:250-258.

162. Ford, H. L., E. N. Kabingu, E. A. Bump, G. L. Mutter, and A. B. Pardee. 1998. Abrogation of the G2 cell cycle checkpoint associated with overexpression of HSIX1: a possible mechanism of breast carcinogenesis. Proc Natl Acad Sci U S A 95:12608-12613.

163. Coletta, R. D., K. Christensen, K. J. Reichenberger, J. Lamb, D. Micomonaco, L. Huang, D. M. Wolf, C. Muller-Tidow, T. R. Golub, K. Kawakami, and H. L. Ford. 2004. The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. Proc Natl Acad Sci U S A 101:6478-6483.

164. Gao, A. C., W. Lou, and J. T. Isaacs. 1998. Down-regulation of homeobox gene GBX2 expression inhibits human prostate cancer clonogenic ability and tumorigenicity. Cancer Res 58:1391-1394.

165. Gao, A. C., W. Lou, and J. T. Isaacs. 2000. Enhanced GBX2 expression stimulates growth of human prostate cancer cells via transcriptional up-regulation of the interleukin 6 gene. Clin Cancer Res 6:493-497.

166. Storti, P., G. Donofrio, S. Colla, I. Airoldi, M. Bolzoni, L. Agnelli, M. Abeltino, K. Todoerti, M. Lazzaretti, C. Mancini, D. Ribatti, S. Bonomini, V. Franceschi, V.

Pistoia, G. Lisignoli, A. Pedrazzini, O. Cavicchi, A. Neri, V. Rizzoli, and N. Giuliani. 2010. HOXB7 expression by myeloma cells regulates their pro-angiogenic properties in multiple myeloma patients. Leukemia Epub 2010 Nov 23.

167. Naora, H., Y. Q. Yang, F. J. Montz, J. D. Seidman, R. J. Kurman, and R. B. Roden. 2001. A serologically identified tumor antigen encoded by a homeobox gene promotes growth of ovarian epithelial cells. Proc Natl Acad Sci U S A 98:4060-4065.

168. Rubin, E., X. Wu, T. Zhu, J. C. Y. Cheung, H. Chen, A. Lorincz, R. K. Pandita, G. G. Sharma, H. C. Ha, J. Gasson, L. A. Hanakahi, T. K. Pandita, and S. Sukumar. 2007. A role for the HOXB7 homeodomain protein in DNA repair. Cancer Res 67:1527-1535.

169. Wu, X., H. Chen, B. Parker, E. Rubin, T. Zhu, J. S. Lee, P. Argani, and S. Sukumar. 2006. HOXB7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial-mesenchymal transition. Cancer Res 66:9527-9534.

170. Quinn, L. M., B. V. Johnson, J. Nicholl, G. R. Sutherland, and B. Kalionis. 1997. Isolation and identification of homeobox genes from the human placenta including a novel member of the Distal-less family, DLX4. Gene 187:55-61.

171. Shimamoto, T., S. Nakamura, J. Bollekens, F. H. Ruddle, and K. Takeshita. 1997. Inhibition of DLX-7 homeobox gene causes decreased expression of GATA-1 and c-myc genes and apoptosis. Proc Natl Acad Sci U S A 94:3245-3249.

172. Haga, S. B., S. Fu, J. E. Karp, D. D. Ross, D. M. Williams, W. D. Hankins, F. Behm, F. W. Ruscetti, M. Chang, B. D. Smith, D. Becton, S. C. Raimondi, and

P. E. Berg. 2000. BP1, a new homeobox gene, is frequently expressed in acute leukemias. Leukemia 14:1867-1875.

173. Hara, F., S. Samuel, J. Liu, D. Rosen, R. R. Langley, and H. Naora. 2007. A homeobox gene related to Drosophila distal-less promotes ovarian tumorigenicity by inducing expression of vascular endothelial growth factor and fibroblast growth factor-2. Am J Pathol 170:1594-1606.

174. Morasso, M. I., R. Yonescu, C. A. Griffin, and T. D. Sargent. 1997. Localization of human DLX8 to chromosome 17q21.3-q22 by fluorescence in situ hybridization. Mamm Genome 8:302-303.

175. Hyman, E., P. Kauraniemi, S. Hautaniemi, M. Wolf, S. Mousses, E. Rozenblum, M. Ringner, G. Sauter, O. Monni, A. Elkahloun, O. P. Kallioniemi, and A. Kallioniemi. 2002. Impact of DNA amplification on gene expression patterns in breast cancer. Cancer Res 62:6240-6245.

176. Man, Y. G., S. W. Fu, A. Schwartz, J. J. Pinzone, S. J. Simmens, and P. E. Berg. 2005. Expression of BP1, a novel homeobox gene, correlates with breast cancer progression and invasion. Breast Cancer Res Treat 90:241-247.

177. Schwartz, A. M., Y.-G. Man, M. K. Rezaei, S. J. Simmens, and P. E. Berg. 2008. BP1, a homeoprotein, is significantly expressed in prostate adenocarcinoma and is concordant with prostatic intraepithelial neoplasia. Mod Pathol 22:1-6.

178. Tomida, S., K. Yanagisawa, K. Koshikawa, Y. Yatabe, T. Mitsudomi, H. Osada, and T. Takahashi. 2007. Identification of a metastasis signature and the DLX4 homeobox protein as a regulator of metastasis by combined transcriptome approach. Oncogene 26:4600-4608.

179. Yu, M., Y. Wan, and Q. Zou. 2008. Prognostic significance of BP1 mRNA expression level in patients with non-small cell lung cancer. Clin Biochem 41:824-830.

180. Bendall, A. J., and C. Abate-Shen. 2000. Roles for Msx and Dlx homeoproteins in vertebrate development. Gene 247:17-31.

181. Germain, S., M. Howell, G. M. Esslemont, and C. S. Hill. 2000. Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. Genes & Dev 14:435-451.

182. Park, G. T., and M. I. Morasso. 2002. Bone morphogenetic protein-2 (BMP-2) transactivates Dlx3 through Smad1 and Smad4: alternative mode for Dlx3 induction in mouse keratinocytes. Nucleic Acids Res 30:515-522.

183. Berghorn, K. A., P. A. Clark-Campbell, L. Han, M. McGrattan, R. S. Weiss, and M. S. Roberson. 2006. Smad6 represses Dlx3 transcriptional activity through inhibition of DNA binding. J Biol Chem 281:20357-20367.

184. Chiba, S., K. Takeshita, Y. Imai, K. Kumano, M. Kurokawa, S. Masuda, K. Shimizu, S. Nakamura, F. H. Ruddle, and H. Hirai. 2003. Homeoprotein DLX-1 interacts with Smad4 and blocks a signaling pathway from activin A in hematopoietic cells. Proc Natl Acad Sci U S A 100:15577-15582.

185. Williams, T. M., M. E. Williams, J. H. Heaton, T. D. Gelehrter, and J. W. Innis. 2005. Group 13 HOX proteins interact with the MH2 domain of R-Smads and modulate Smad transcriptional activation functions independent of HOX DNA-binding capability. Nucleic Acids Res 33:4475-4484.

186. Li, X., S. Nie, C. Chang, T. Qiu, and X. Cao. 2006. Smads oppose Hox transcriptional activities. Exp Cell Res 312:854-864.

187. Fu, Y., Y. Lian, K. S. Kim, L. Zhang, A. K. Hindle, F. Brody, R. S. Siegel, T. A. McCaffrey, and S. W. Fu. 2010. BP1 Homeoprotein enhances metastatic potential in ER-negative breast cancer. J Cancer 1:54-62.

188. Cavalli, L. R., Y. G. Man, A. M. Schwartz, J. D. Rone, Y. Zhang, C. A. Urban, R. S. Lima, B. R. Haddad, and P. E. Berg. 2008. Amplification of the BP1 homeobox gene in breast cancer. Cancer Genet Cytogenet 187:19-24.

189. Kluk, B. J., Y. Fu, T. A. Formolo, L. Zhang, A. K. Hindle, Y. G. Man, R. S. Siegel, P. E. Berg, C. Deng, T. A. McCaffrey, and S. W. Fu. 2010. BP1, an isoform of DLX4 homeoprotein, negatively regulates BRCA1 in sporadic breast cancer. Int J Biol Sci 6:513-524.

190. Stevenson, H., S. Fu, J. Pinzone, J. Rheey, S. Simmens, and P. Berg. 2007. BP1 transcriptionally activates bcl-2 and inhibits TNFalpha-induced cell death in MCF7 breast cancer cells. Breast Cancer Res Treat 9:R60.

191. Quinn, L. M., S. E. Latham, and B. Kalionis. 1998. A distal-less class homeobox gene, DLX4, is a candidate for regulating epithelial-mesenchymal cell interactions in the human placenta. Placenta 19:87-93.

192. Sun, Y., X. Lu, L. Yin, F. Zhao, and Y. Feng. 2006. Inhibition of DLX4 promotes apoptosis in choriocarcinoma cell lines. Placenta 27:375-383.

193. He, T. C., A. B. Sparks, C. Rago, H. Hermeking, L. Zawel, L. T. da Costa, P. J. Morin, B. Vogelstein, and K. W. Kinzler. 1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509-1512.

194. Zawel, L., J. L. Dai, P. Buckhaults, S. Zhou, K. W. Kinzler, B. Vogelstein, and S. E. Kern. 1998. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell 1:611-617.

195. Tournay, O., and R. Benezra. 1996. Transcription of the dominant-negative helix-loop-helix protein Id1 is regulated by a protein complex containing the immediate-early response gene Egr-1. Mol Cell Biol 16:2418-2430.

196. Korchynskyi, O., and P. ten Dijke. 2002. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883-4891.

197. Satterwhite, D., M. Aakre, A. Gorska, and H. Moses. 1994. Inhibition of cell growth by TGF beta 1 is associated with inhibition of B-myb and cyclin A in both BALB/MK and Mv1Lu cells. Cell Growth Differ 5:789-799.

198. Reynisdottir, I., K. Polyak, A. Iavarone, and J. Massague. 1995. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes & Dev 9:1831-1845.

199. Moustakas, A., K. Pardali, A. Gaal, and C.-H. Heldin. 2002. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol Lett 82:85-91.

200. Deckers, M., M. van Dinther, J. Buijs, I. Que, C. Lowik, G. van der Pluijm, and P. ten Dijke. 2006. The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res 66:2202-2209.

201. Shi, Y., and J. Massague. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685-700.

202. Kusanagi, K., H. Inoue, Y. Ishidou, H. K. Mishima, M. Kawabata, and K. Miyazono. 2000. Characterization of a bone morphogenetic protein-responsive Smad-binding element. Mol Biol Cell 11:555-565.

203. Miyazawa, K., M. Shinozaki, T. Hara, T. Furuya, and K. Miyazono. 2002. Two major Smad pathways in TGF-beta superfamily signalling. Genes to Cells 7:1191-1204.

204. Shen, W. F., S. Rozenfeld, A. Kwong, L. G. Kom ves, H. J. Lawrence, and C. Largman. 1999. HOXA9 forms triple complexes with PBX2 and MEIS1 in myeloid cells. Mol Cell Biol 19:3051-3061.

205. Boogerd, K. J., L. Y. Wong, V. M. Christoffels, M. Klarenbeek, J. M. Ruijter, A. F. Moorman, and P. Barnett. 2008. Msx1 and Msx2 are functional interacting partners of T-box factors in the regulation of Connexin43. Cardiovasc Res 78:485-493.

206. Satoh, Y., I. Matsumura, H. Tanaka, S. Ezoe, H. Sugahara, M. Mizuki, H. Shibayama, E. Ishiko, J. Ishiko, K. Nakajima, and Y. Kanakura. 2004. Roles for c-Myc in self-renewal of hematopoietic stem cells. J Biol Chem 279:24986-24993.

207. Daksis, J. I., R. Y. Lu, L. M. Facchini, W. W. Marhin, and L. J. Penn. 1994. Myc induces cyclin D1 expression in the absence of de novo protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. Oncogene 9:3635-3645.

208. Wierstra, I., and J. Alves. 2008. The c-myc promoter: still MysterY and challenge. Adv Cancer Res 99:113-333.

209. McGinnis, W., and R. Krumlauf. 1992. Homeobox genes and axial patterning. Cell 68:283-302.

210. Yang, X., X. Ji, X. Shi, and X. Cao. 2000. Smad1 domains interacting with Hoxc-8 induce osteoblast differentiation. J Biol Chem 275:1065-1072.

211. Tokunaga, H., D. H. Lee, I. Y. Kim, T. M. Wheeler, and S. P. Lerner. 1999. Decreased expression of transforming growth factor beta receptor type I is associated with poor prognosis in bladder transitional cell carcinoma patients. Clin Cancer Res 5:2520-2525.

212. Gemma, A., S. Takenoshita, K. Hagiwara, A. Okamoto, E. A. Spillare, M. G. McMemamin, S. P. Hussain, K. Forrester, M. Zariwala, Y. Xiong, and C. C. Harris. 1996. Molecular analysis of the cyclin-dependent kinase inhibitor genes p15INK4b/MTS2, p16INK4/MTS1, p18 and p19 in human cancer cell lines. Int J Cancer 68:605-611.

213. Rapp, U. R., C. Korn, F. Ceteci, C. Karreman, K. Luetkenhaus, V. Serafin, E. Zanucco, I. Castro, and T. Potapenko. 2009. MYC is a metastasis gene for non-small-cell lung cancer. PLoS One 4:e6029.

214. Watanabe, T., I. Imoto, Y. Kosugi, I. Ishiwata, S. Inoue, M. Takayama, A. Sato, and J. Inazawa. 2001. A novel amplification at 17q21-23 in ovarian cancer cell lines detected by comparative genomic hybridization. Gynecol Oncol 81:172-177.

215. Zhang, X., T. Zhu, Y. Chen, H. C. Mertani, K. O. Lee, and P. E. Lobie. 2003. Human growth hormone-regulated HOXA1 is a human mammary epithelial oncogene. J Biol Chem 278:7580-7590.

216. Care, A., A. Silvani, E. Meccia, G. Mattia, A. Stoppacciaro, G. Parmiani, C. Peschle, and M. P. Colombo. 1996. HOXB7 constitutively activates basic fibroblast growth factor in melanomas. Mol Cell Biol 16:4842-4851.

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

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2010	Schissler Foundation Fellowship in the Genetics of Human Disease
2009	Trainee Excellent Award, MD Anderson Cancer Center
2009	Vietnam Education Foundation International Conference Travel Grant
2009	GSBS international meeting travel award, Graduate School of Biomedical Sciences
2009	Best Poster Presentation Award, 6th Annual Vietnam Education Foundation Conference
2008	Student Presentation Award, UT Health Sciences Center, Graduate School of Biomedical Sciences
2007	Graduate Teaching Assistant Award, Graduate School of Biomedical Sciences
2006-2008	Professional Development Grant, Vietnam Education Foundation
2005	Best presentation award, Biological and Chemistry scientific session, 2nd Vietnam Education Foundation Annual Conference
2004-2011	Vietnam Education Foundation Fellowship
2004	First place in the entrance examination of JETC fellowship for Ph.D study in Greifswald/Germany, Joint Educational Training Center Hanoi-Greifswald
2001	Tokyo-Mitsubishi Bank undergraduate study scholarship
1998-2002	Vietnam Ministry of Education & Training Scholarship

Publication

1. **Trinh BQ**, Barengo N and, Naora H. Homeodomain protein DLX4 counteracts key transcriptional control mechanisms of the TGF-β cytostatic program and blocks the anti-proliferative effect of TGF-β. Oncogene. 2011, Feb 7. PMID: 21297662
2. Xie X, Hsu JL, Choi MG, Xia W, Yamaguchi H, Chen CT, **Trinh BQ**, Lu Z, Ueno NT, Wolf JK, Bast RC Jr, Hung MC. A novel hTERT promoter–driven E1A therapeutic for ovarian cancer. Mol Cancer Ther, 2009 Aug;8(8):2375-82. PMID: 19671744
3. Vu BTN, Nguyen DK, **Trinh BQ**, Bach QTN Dinh KD, Dinh VT. Detection and quantification of yellow head virus load in shrimp using Real Time PCR with SYBR Green. Journal of Science (Hanoi National University) 2005, 21(2): 59-65. (In Vietnamese)
4. Le CTM, Dai BD, Hoang CM, Nguyen VV, Le DT, Dinh KD, Bach QTN, **Trinh BQ**, Duong QH, Nguyen HH, Nguyen DT, Nguyen DB.. Detection of BRCA1 and BRCA2 mutations in breast cancer patients by Real time PCR. National Conference on Basic Reseach. Institute of Military Medicine, October 28, 2004: 372-376. (In Vietnamese)
5. **Trinh BQ**, Nguyen BH, Truong NU, Le MTQ, Nguyen HTH, Dinh KD. 2003. Cloning and expression the region encoding Premembrane and envelope proteins of dengue virus type 4. Journal of Biotechnology 2003, 1(1): 33-38. (In Vietnamese)

Selected conference presentations

1. Blockade of transforming growth factor-b signaling in tumors by the homeobox patterning gene DLX4, **Trinh BQ**, Barengo N and Naora H, Keystone Symposia, Vancouver, Canada, 2011
2. The role of homeobox gene DLX4 in angiogenesis of ovarian cancer, **Trinh BQ**, Barengo N and Naora H, 2nd World Cancer Congress, Beijing, China, 2009
3. Novel function of homeobox gene DLX4 in regulating tumor angiogenesis, **Trinh BQ**, Barengo N and Naora H, The American Association for Cancer Research (AACR) 100th Annual Meeting, Denver, Colorado, 2009
4. Enhanced hTERT promoter and its potential application for ovarian cancer gene therapy, **Trinh BQ**, Xie X, Hung MC, 4th Vietnam Education Foundation Annual Conference, California, 2008
5. Generation of Premembrane and envelope recombinant antigens from dengue virus type 4 and development of diagnostic kits for Dengue fever and dengue hemorrhagic fever, **Trinh BQ** and Dinh KD, National Microbiology conference, Dalat, Vietnam, 2003
6. Cloning of the gene coding for enterotoxin subunit A from Vibrio cholerae serotype Inaba for development of cholera diagnostic kit, **Trinh BQ** and Dinh KD, Scientific research symposium, Faculty of Biology, Hanoi College of Science, Vietnam National University, 2002

EMBL-EBI and NCBI Gene databases

1. **Trinh BQ**, Truong NU, Truong TT, Le QTM, Nguyen HTH and Dinh KD. Cloning and expression of the genes encoding PreM and Envelope proteins of of Dengue virus type 4. Access No: AJ563356. (13-MAY-2003). Related news covered by

Vietnam National media VietNamNet: Development of the diagnostic Kit for rapid detection of Dengue virus

2. Ha QT, **Trinh BQ**, Bach QTN, Phung CD, Do KN and Dinh KD. Cloning of the gene coding for enterotoxin subunit A from Vibrio cholerae serotype Inaba. Access No: AJ575590. (11-JUL-2003)
3. Ghazi AK, **Trinh BQ**, Truong DV and Dinh KD. Cloning and expression of the gene encoding VP2 protein of Infectious Bursal Disease virus. Access No: AJ577092. (15-JUL-2003)
4. Bui HT, Vu HT, Le HL, **Trinh BQ**, Nguyen MT, Bach QTN, Nguyen CT, Dinh KD. Cloning of the gene coding for homoserine acetyltransferase from Corynebacterium glutamicum isolate TK3600. EMBL/GenBank/DDBJ databases. Access No: AJ584663. (04-OCT-2003)