THE SMAD3 LINKER REGION: TRANSCRIPTIONAL ACTIVITY AND PHOSPHORYLATION-MEDIATED REGULATION

by

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ABSTRACT OF THE DISSERTATION THE SMAD3 LINKER REGION: TRANSCRIPTIONAL ACTIVITY AND PHOSPHORYLATION-MEDIATED REGULATION By GUANNAN WANG Dissertation Director:

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TGF- β regulates cell proliferation, differentiation, apoptosis, and extracellular matrix production. Smad proteins are central mediators of TGF- β signaling. Upon ligand binding, Smad2 and Smad3 are phosphorylated by the receptor at the C-terminal SxS motif. The phosphorylation triggers heteromeric complex formation with Smad4 and the translocation of the Smads into the nucleus, where they recruit co-activators, corepressors and other transcription factors to activate or repress transcription of target genes.

The Smad3 linker region, a motif of 90 amino acid residues located between the Nterminal and C-terminal domains of Smad3, contains several serine/threonine-proline motifs, which are putative phosphorylation sites for proline-directed protein kinases such as Mitogen-Activated Protein Kinase (MAPK) family members and cyclin-dependent kinases (CDKs). We have shown that the Extracellular-signal Regulated Kinase (ERK) MAPK phosphorylates three linker sites Thr 179, Ser 204, and Ser 208 of Smad3, and that G1 CDKs phosphorylate Thr 179 and Ser 213 in the linker region and Thr 8 in the Nterminal domain of Smad3. Phosphorylation of Smad3 by CDK and ERK inhibits its transcriptional activity. In addition, TGF-β induces Smad3 phosphorylation on Thr 179, Ser 204, and Ser 208 in a C-tail phosphorylation-dependent manner, and the phosphorylation appears to be inhibitory. We have found that the linker region of Smad3 contains a transcriptional activation domain. Previous studies showed that the C-terminal domain of Smad3 is essential for Smad transcriptional activation through its interaction with the co-activator p300. We found that the Smad3 linker region can also interact with the co-activator p300. Deletion of the Smad3 linker region from the full-length protein abolished the ability of Smad3 to activate several TGF- β responsive reporter genes. We further showed that the linker region and the C-terminal domain of Smad3 synergize for transcriptional activation in the presence of TGF-β. In addition, mutation of the CDK and ERK phosphorylation sites in Smad3 increases its ability to interact with p300. This suggests that CDK and ERK phosphorylation of Smad3 inhibits its binding to p300. Since cancer cells often contain high levels of CDK and ERK activity, CDK and ERK phosphorylation of Smad3 may contribute to tumorigenesis and TGF- β resistance in cancers.

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Chapter I: Introduction

1.1 TGF- β superfamily

The Transforming Growth Factor β (TGF- β) superfamily is a group of sequencerelated, structurally-similar growth and differentiation factors that are conserved in mammals, *Drosophila*, and *C. elegans*. It includes TGF- β s, Bone Mophorgenetic Proteins (BMPs), Activins, Inhibins, Growth and Differentiation Factors (GDFs), and Mullerian Inhibiting Substance (MIS) [1-5]. There are totally 42 proved or predicted members of TGF- β superfamily in human, 9 in *Drosophila*, and 6 in *C. elegans* [4]. TGF- β ligands play essential roles in embryonic development, tissue homeostasis, and tumorigenesis [6, 7].

TGF- β , the founding member of the TGF- β superfamily, regulates cell proliferation, differentiation, apoptosis, motility and extracellular matrix production. TGF- β strongly inhibits growth of epithelial, endothelial, neuronal, and immune cells. Therefore TGF- β suppresses tumor formation at initial stage. However, TGF- β promotes tumorigenesis in later stages by stimulating cancer cell motility and invasion, fostering tumor angiogenesis, inhibiting immune survaillence and promoting metastasis [8, 9].

TGF- β ligands are secreted molecules that contain 7 conserved signature cysteines in their primary sequence. Two TGF- β polypeptides form a dimer that is joined by one inter-subunit disulfide bond. The remaining 6 conserved cysteines in each subunit form three pairs of intra-subunit disulfide bonds that interlock several β -strands together to form a tight structure termed the "cysteine knot" [4]. TGF- β is synthesized as a ~400 aa precursor that is subsequently cleaved by the protease furin to yield mature TGF- β , which is still non-covalently bound by the pro-peptide. Such TGF- β is biologically inert and requires the removal of pro-peptide to become active [5].

1.2 TGF- β superfamily signaling

1.2.1 Overview of TGF- β /Smad signaling

TGF- β ligands signal through the type II and type I transmembrane serine/threonine kinase receptors. The type II receptor kinase is constitutively active. Ligand binding induces the formation of type II/type I receptor complex, in which type II receptor phosphorylates type I receptor, leading to the activation of type I receptor. Type I receptor then phosphorylates receptor-regulated Smad proteins (R-Smads), the essential intracellular mediators of TGF- β superfamily signaling. Phosphorylated R-Smad associates with the common mediator Smad (Co-Smad, i.e. Smad4), and together they translocacte in the nucleus, where they associate with other transcription factors, coactivators, and co-repressors to activate or suppress the expression of target genes (Figure 1.1) [5]. Smad pathway plays a major role in transducing TGF- β signals, although Smad-independent pathways can emanate from activated type II/type I receptor complex [1].

1.2.2 The receptors

Two types of receptors, the type II and the type I receptors, are required for the signaling of TGF- β ligands. Both types of receptors are transmembrane serine/threonine kinases that possess extracellular ligand binding domain, transmembrane domain, and the intracellular kinase domain. The type II receptor is constitutively active, while the type I receptor's activity is regulated through phosphorylation by the type II receptor on a juxtamembrane TTSGSGSG motif termed the "GS" domain. Ligand binds to the type



Figure 1.1 Overview of TGF- β /Smad signaling, adapted from [3, 5]. TGF- β ligands signal through the transmembrane serine/threonine kinases the type II and type I receptors. The type II receptor is constitutively active. Upon ligand binding, type II receptor and type I receptor form a complex in which the type II receptor phosphorylates the type I receptor. The type I receptor then phosphorylates the intracellular receptor-regulated Smad (R-Smad). Phosphorylated R-Smad then form a complex with co-Smad Smad4, and together they accumulate in the nucleus, where they recruit co-activator, co-repressor or other transcription factors to regulate target gene expression.

II and the type I receptors and brings together the intracellular kinase domains of type I and type II receptors, triggering the phosphorylation of type I receptor GS domain by type II receptor. This leads to type I receptor activation [5]. Although the number of ligands is large, their signals converge on 5 type II (T β RII, ActRII, ActRIIB, BMPRII, MISRII) and 7 type I receptors (T β RI, ActRI, ActRIB, BMPRIA, BMPRIB, ALK1, and ALK7; also systematically named ALK1~ALK7) through different type II-type I receptor combinations (Table 1.1). Of the two receptors, type I receptor plays an important role in transducing the signal further downstream and determining signaling specificity. BMP ligands bind to BMP type I receptors (ALK1, ActRI, BMPRIA, BMPRIA, BMPRIB), while TGF- β /activin signals through TGF- β or activin type I receptors (T β RI, ActRIB, ALK7) (Table 1.1) [1].

1.2.3 Mechanisms of ligand binding

Since the ligands are dimers, the ligand-receptor complex likely contains a dimeric ligand, two type II receptors and two type I receptors. Two modes of ligand binding to receptors can occur depending on the different affinities of ligands toward type II or type I receptor. BMPs exhibit higher affinity for the type I receptor's ligand binding domain. BMPs first bind to type I receptor, and the pre-assembled type I receptor-BMP complex then recruits the low affinity type II receptor (Figure 1.2). On the contrary, TGF- β s and activins have higher affinity for their type II receptors. Therefore, TGF- β and activins bind type II receptor first and subsequently recruit the type I receptor into the complex (Figure 1.2) [4]. Ligand binding is regulated by soluble ligand binding proteins, co-receptors, and receptor accessory proteins. Extracellular regulators of TGF- β super-

Ligand	Type II receptor	Type I receptor	Smad
TGF-β1	TβRII	TβRI (ALK5)	Smad2/3
TGF-β2 TGF-β3		ALK1	Smad1/5/8
Activin Nodal	ActRII ActRIIB	ActRIB (ALK4) ALK7	Smad2/3
BMP2/4 BMP6 BMP7	ActRII ActRIIB BMPRII	ALK1 ActRI (ALK2) BMPRIA (ALK3) BMPRIB (ALK6)	Smad1/5/8

Table 1.1 Components and specificity of TGF-β superfamily pathways

This table is based on reference [1], Abbreviations: ALK: <u>A</u>ctivin-receptor <u>Like K</u>inase; the type II receptors are named by their ligand, e.g. T β RII for TGF- β , BMPRII for BMP, but ActRII and ActRIIB can bind both activin and certain BMPs. Alternative names for the type I receptor ALKs are: ALK1, ACVRL1 (<u>ACtiVin Receptor Like-1</u>); ALK2, Acvr1, ACVRI, ActRIA; ALK3, BMPRIA, Bmpr1a; ALK4, ACVR1B, Acvr1b, ActRIB; ALK5, TGFBR1; ALK6, BMPRIB, Bmpr1b; ALK7, ACVRIC, Acvr1c.



Figure 1.2 Two modes of ligand binding to receptors of the TGF- β superfamily, based on references [4, 5]. Upper, TGF- β /activin first bind to type II receptor, then the type II receptor-ligand complex recruits the type I receptor to form the ternary complex. Lower, BMP ligands have higher affinity for and first bind to type I receptor, then the type II receptor joins the complex.

family ligands binding are summarized in Table 1.2.

1.2.4 The Smads

Smad proteins were first identified in genetic screens for modifiers of the Drosophila Decapentaplegic (Dpp) pathway [10] and genes responsible for the *C.elegans* "Small" phenotype [11]. Receptor-regulated Smads (R-Smad) are substrates of the activated type I receptor kinase [12-15]. Interaction between the L3 loop of Smad3 and L45 loop in the kinase domain of type I receptor determines signaling specificity. Smad1, 5, 8 interact with and are phosphorylated by BMP type I receptors to transduce BMP signals [1, 13, 16-21], whereas Smad2 and Smad3 are phosphorylated by TGF-β or activin type I receptors to relay TGF-β/activin signals (Table 1.1) [1, 14, 15, 22, 23]. Another class of Smad, called Co-Smad, associates with R-Smads upon their phosphorylation and is essential for downstream function of R-Smads [1, 24, 25]. The only mammalian Co-Smad is Smad4. Co-Smad is shared by both the BMP branch and the TGF-β/activin branch of signaling [25]. A third class of Smad is inhibitory Smads (I-Smads) which include Smad6 and Smad7 [1, 5, 26-32]. They act as inactive decoys to bind to type I receptor and competitively repress R-Smad phosphorylation. Smad6 can also interact with Smad1 to form an inactive Smad1/Smad6 complex, thereby suppressing the formation of the functional Smad1/4 complex. Smad7 inhibits the signaling of both TGF- β /activin and BMP, but Smad6 preferentially represses BMP signal transduction (Figure 1.3).

Ligand	Regulator	Effect	Mechanism
TGF-β	Latency Associated Protein (LAP) Decorin	inhibition	ligand binding and sequestration
	Betaglycan Endoglin	activation	co-receptors, assist for ligand binding to type II and type I receptors
	Thrombospondin-1 Integrin αvβ6	activation	latent TGF-β activation
Nodal	Cripto Criptic	activation	accessory protein for ligand binding
BMP	noggin Chordin (Sog) DAN family	inhibition	ligand binding and sequestration, they are called "ligand sink"
	Tolloid (Drosophila) Xolloid (Xenopus) BMP-1 (human) hTld1 (human)	activation	cleaving chordin thereby releafing BMPs from "ligand sink"

Table 1.2 Extracellular regulators of TGF- β superfamily ligands

This table is based on reference [2, 3], Abbreciations: Xolloid, Xenopus Tolloid; hTld1, human Tolloid-1; LAP, Latency Associated Protein.



Figure 1.3 Three classes of Smads. Adapted from [5]. Two branches of R-Smads, those transducing TGF- β /activin signals (Smad2/3) and those transducing BMP signals (Smad1/5/8), are present in mammals. Two R-Smads in *Drosophila*, dSmad2 and Mad, transduce signals from different *Drosophila* TGF- β like pathways. The only Co-Smad in mammals is Smad4. However, there are two Co-Smads in *Xenopus*: Smad4 α (Smad4) and Smad4 β . R-Smads, after being activated by type I receptor, associate with Co-Smad. Together, the R-Smad-Co-Smad complex translocates into the nucleus and regulates target gene transcription. I-Smads act as inactive decoys for type I receptor binding and competitively inhibit type I receptor phosphorylation of R-Smads. I-Smad expression is stimulated by TGF- β and BMP ligand stimulation, forming a negative feedback regulatory loop in signaling of TGF- β ligands.

1.2.5 Structure-function relationship of Smad domains

Smads contain two conserved domain, the N-terminal MH1 (Mad Homology 1) domain and the C-terminal MH2 (Mad homology 2) domain. MH1 and MH2 domains are joined together by a less conserved linker region (Figure 1.4). The MH1 domains of R-Smad and Co-Smad possess DNA binding activity [5, 33, 34], whereas the MH2 domain together with part of the linker region can associate with transcriptional coactivators and can transactivate reporter gene when fused to GAL4 DNA binding domain [5, 35-41]. The L3 loop of MH2 domain bears determinants for pathway specificity [42-44], whereas the C-terminus of R-Smad is the conserved SSxS motif that is phosphorylated by the type I receptor [13-15, 22, 23]. The linker region of R-Smad contains demonstrated as well as suspected phosphoryaltion sites by multiple kinases, e.g. MAPK, CDK, CamKII, etc [45-50, 151]. The linker region of R-Smad and I-Smad contains a PPxY motif which can be bound by the WW domains of E3 ubiquitin ligase Smurf. Smad2/3 recruit the E3 ubiquitin ligase Smurf2 to degrade the co-repressor SnoN [52], whereas the I-Smads Smad6 and Smad7 recruit Smurf1/2 to target the BMP and TGF- β type I receptors for degradation [53-55].

1.2.6 R-Smad phosphorylation by the type I receptor

R-Smad and the type I receptor interact through their L3 and L45 loops, respectively [43, 44]. A highly basic surface region near L3 loop on R-Smad can additionally interact with the phosphorylated GS domain of the type I receptor [56]. In the basal state, the protein FKBP12 binds the unphosphorylated GS domain, locking the kinase domain in an inactive and inaccessible conformation. Upon ligand binding, the GS domain is



Figure 1.4 R-Smad domain structure and functions, adapted from [3, 4]. R-Smad comprises three domains, the highly conservative N-terminal Mad Homology 1 (MH1) domain and C-terminal Mad homology 2 (MH2) domain and a linker region that is different in length and sequence among different Smads. The MH1 domain can bind DNA through a highly conserved β -hairpin structure. MH1 domain also binds to Smad DNA binding partners (co-factors). In addition, several regulatory phosphorylation sites by CDK, CamKII and PKC reside in the MH1 domain. The MH2 domain of R-Smad is a versatile protein-protein interaction domain. MH2 domain mediates interaction with type I receptor and Smad Anchor for Receptor Activation (SARA), which is essential for the ligand induced C-tail phosphorylation. MH2 domain mediates R-Smad homooligomerization and R-Smad-Co-Smad hetero-oligomerization. MH2 domain binds coactivators and co-repressors and mediates activation or repression of target genes. MH2 domain also contains structure determinants that bind many other transcription factors in cooperative gene regulation. The linker region of Smad3 contains a ligand-independent transcription activation domain. The linker region of R-Smad also contains several regulatory phosphorylation sites that are targets of Mitogen-Activated Protein Kinase (MAPK), cyclin dependent kinases (CDK), and Ca²⁺/Calmodulin Dependent Protein Kinase II (CamKII). Linker regions of most Smads also contain the PPxY motif which is the binding motif for WW domains of ubiquitin E3 ligases Smurf1/2.

phosphorylated and the FKBP12 dissociates, allowing R-Smad to bind. Therefore, FKBP12 prevents opportunistic activation of R-Smad by type I receptor in the absence of ligand [57, 58].

Smad2/3 are presented to the TGF- β type I receptor by Smad Anchor for Receptor Activation (SARA) [56, 59]. SARA is a FYVE domain containing protein that is localized to endosomes and interacts with both Smad2/3 and type I receptor, bringing the two together to facilitate the phosphorylation reaction. SARA has two additional functions: first, Smad2/3 binding by SARA prevents them from oligomerization, thereby preventing aberrant Smad activation in the basal state; Second, SARA binding keeps a significant portion of Smad2/3 in the cytoplasm in the un-stimulated state, which guarantees that they can respond to receptor activation quickly [59, 60]. The cytoplasmic form of tumor suppressor ProMyelocytic Leukemia (cPML) interacts with both TGF- β receptors and Smad2/3. cPML enhances SARA-Smad interaction, assisting SARA to execute its essential function as an R-Smad-receptor adaptor [61]. Many other adaptor molecules have been identified that play a similar role as SARA and cPML, i.e. bridging the R-Smad-type I receptor interaction and facilitating receptor mediated phosphorylation of R-Smad [reviewed in 62]. Adaptor proteins that facilitate receptor mediated Smad phosphorylation and downstream signaling are summarized in Table 1.3.

Phosphorylation of Smad2, and most likely other R-Smads, occurs on the last two serines (S465, S467 in Smad2) of the SSxS motif [22, 23]. Although S464 of Smad2 itself is not phosphorylated, it is required for S465/467 phosphorylation. Furthermore, S465/467 phosphorylation occurs in an obligate order: S467 phosphorylation is required for S465 to be phosphorylated. It seems that C-tail singly phosphorylated at S467 is

Protein	Receptor Interaction	Smad Interaction	Mechanisms
SARA	TBRI/II	Smad2/3	Present Smad2/3 to TBRI for
57 1107	1 pro/ 11	5111442/5	phosphorylation
Hrs	presumably TβRI/II	Smad2	Assist in Smad2/3-receptor recognition and receptor-mediated Smad2/3 phosphory- lation
cPML	TβRI/II	Smad2/3	Enhance SARA-Smad2/3 interaction, required for receptor mediated Smad2/3 phosphorylation
Dab2	TβRI/II	Smad2/3	Enhance TβRI mediated Smad phosphorylation
Axin	ΤβRΙ	Smad2/3	Enhance TGF-β stimulated Smad2/3 signaling
ELF	ΤβRΙ	Smad3/4	Phosphorylated by receptors and associate with Smad3/4 upon ligand stimulation
TRAP-1	ΤβRΙ	Smad4	Facilitate Smad4 association with Smad2 upon TβRI mediated Smad2
TLP	TβRII	Smad4	phosphorylation Interfere with Smad3-Smad4 interaction but augment Smad2 signaling

Table 1.3 Intracellular adaptor and scaffolding proteins for Smad phosphorylation

This table is adapted from reference [62]. Abbreviations are: T β RI, <u>T</u>GF-<u> β </u> <u>Receptor</u> Type I; T β RII, <u>T</u>GF-<u> β </u> <u>Receptor</u> Type II; SARA, <u>Smad</u> <u>Anchor for</u> <u>Receptor</u> <u>Activation</u>; Hrs, <u>Hepatocyte</u> growth factor-<u>regulated</u> tyrosine kinase <u>substrate</u>, also called Hgs; cPML, the <u>cytoplasmic form of ProMyelocytic</u> <u>Leukemia tumor</u> suppressor protein; Dab2, <u>Disabled</u> 2; ELF, <u>Embryonic</u> <u>Liver</u> <u>Fodrin</u>; TRAP-1, <u>T</u>GF-<u> β </u> <u>Receptor</u> <u>Associated</u> <u>Protein-1</u>; TLP, <u>T</u>RAP-1 <u>Like</u> <u>Protein</u>.

more easily recognized by the kinase than the unphosphorylated C-tail [22, 63]. Sequential phosphorylation of S467 and S465 will guarantee that the C-tail phosphorylation occurs only when the type I receptor kinase activity reaches a threshold level.

After R-Smad is phosphorylated by the type I receptor, it dissociates from receptor and translocates into the nucleus, where it fulfills its transcription function [12-14]. Mutation analysis has demonstrated that C-tail phosphorylation is required for R-Smad to dissociate. Wild type Smad2 can stably associate with a kinase defective type I receptor, but it only transiently interacts with kinase active type I receptor. When the serines in the R-Smad C-terminal motif are mutated to non-phosphorylatable alanines or phosphorylation mimicking aspartic acids, the resulting R-Smads constitutively associate with TβRI [22]. This suggests that negative charge on the aspartic acid substituted C-tail is not sufficient for R-Smad-receptor dissociation.

In the basal state, the MH1 domain and MH2 domain of R-Smad and Smad4 interact with each other and inhibit each other's function [64]. That is, the MH2 domain inhibits MH1 domain's DNA binding activity and the MH1 domain inhibits the MH2 domain's transcription activity. Phosphorylation of the C-terminal SSxS motif of R-Smad relieves the mutual inhibition of Smad MH1 and MH2 domains. Phosphorylation induced Smad conformational change therefore reset R-Smad in a state competent for subsequent functioning in the nucleus.

C-tail phosphorylation disrupts Smad2/3-SARA interaction [59]. Structure studies revealed that SARA bound Smad3 MH2 domain have a different subdomain arrangement compared to the activated Smad3 MH2 domain, and that the conformation

of phosphorylated MH2 domain is incompatible with SARA binding [65]. This suggests that SARA preferentially recognizes the basal state, un-phosphorylated Smad, and upon R-Smad phosphorylation, SARA dissociates from R-Smads to allow them to function in the nucleus.

1.2.7 Activation induced oligomerization of R-Smads and Co-Smad

After phosphorylation of R-Smad on the C-terminal motif it dissociates from the receptor kinase and SARA. The phospho-C-tail can associate with the L3 loop of another R-Smad or Smad4 [66, 67, 73]. In addition, Smad2, and presumably other R-Smad, contain an extended basic region near the L3 loop. This basic region reinforces the phospho-C-tail-L3 loop interaction. Interaction of the phosphorylated C-tail of one R-Smad with the MH2 domain of another R-Smad or Smad4 results in Smad homo- or hetero-oligomerization.

Biochemical fractionation studies revealed that R-Smad preferentially exists as monomers in the basal state [68]. It has also been found that Smad1 and Smad3 have the propensity to trimerize when protein concentration increases. Mutation of C-terminal serines to phospho-mimicking residues greatly increased trimerization. Both structure and biochemical studies demonstrated that pseudo-phosphorylated Smad1, Smad3 and phosphorylated Smad2 form trimers using conserved trimer interface [65, 67-69]. These observations suggest that R-Smads undergo phosphorylation dependent monomer-trimer transition upon activation.

The Smad4 MH2 domain plus part of the linker region exists as trimers in crystal structures [72] and many tumor-derived mutations map to the trimer interface residues

that mediate subunit-subunit interaction [71]. This suggests that the trimeric arrangement of Smad4 is biologically relevant. However, on size exclusion chromatography, Smad4 (AF) (Smad4 MH2 domain plus Smad Activation Domain (SAD), which is part of the linker region) elutes as monomers. In cells, Smad4 can exist either as monomers or trimers in the basal state, revealed by studies conducted in different cell lines [68, 74].

The formation of R-Smad-Smad4 hetero-complex is essential for TGF-β ligands signaling. R-Smad-Smad4 hetero-oligomerization is mediated through similar trimer interfaces as those used in their homo-oligomerization. A key interaction is between the phospho-C-tail of an R-Smad and the L3 loop region of Smad4 or another R-Smad. Size exclusion fractionation of a mixture of Smad4 (AF) and pseudo-phosphorylated (i.e., those substituted at C-terminus by phospho-mimicking amino acid residues) Smad1/3LC (linker plus MH2 domain) demonstrated that Smad4-Smad1/3 form 1:2 ratio complex [69]. However, the phospho-Smad2-Smad4 complex was controversially reported in different studies to be either heterodimer or heterotrimer [70, 75].

What is the molar ratio of R-Smad and Smad4 in transcription complex bound to DNA elements in target promoters? In one study [76], Smad2/3 or Smad4 bearing different tags were used in electrophoresis mobility shift assay (EMSA) using activin response element (ARE) from Mix.2 promoter and Smad binding element (SBE) in c-Jun promoter as probes. The stoichiometry of Smad/DNA complex was investigated by supershift assay by antibodies of different tags. It was demonstrated that the complex assembled on Mix.2 ARE contains two Smad2, one Smad4, and one Forkhead activin signal transducer (Fast) 1/3, whereas one Smad3, one Smad4 and two additional

components are bound to c-Jun SBE. Therefore, the components of Smad complexes may depend on individual promoter contexts.

1.2.8 Nuclear-cytoplasmic shuttling of R-Smad and Co-Smad

The nuclear pore complex (NPC) mediates the transport of proteins between cytoplasm and nucleus. Nuclear import of signaling molecules and transcription factors can occur through direct interaction with NPC component nucleoporins or via adaptor molecules such as importin α and importin β [77].

Both R-Smad and Smad4 translocate into the nucleus upon ligand stimulation, but different R-Smad and Smad4 do this through distinct structural element (see Table 1.4 for a summary). All R-Smads contain a conserved basic rich region (KKLKK) in the MH1 domain. In Smad1 and Smad3, this motif has been demonstrated to act as an NLS (Nuclear Localization Signal) and mediate their ligand induced nuclear translocation in an importin β dependent manner [80-82]. Interaction of Smad3 with importin β is induced by TGF- β . In Smad2, however, this NLS is not functional due to the nearby insertion of its unique exon 3 sequence. The R-Smad NLS is not completely conserved in Smad4 due to a lysine to glutamate substitution. Therefore, the Smad4 counterpart of R-Smad NLS is not functional by itself. Instead, this motif together with other basic residues in Smad4 MH1 domain, mediate Smad4 nuclear import via interaction with importin α [79].

In an in vitro nuclear import assay using digitonin permeabilized cells, the MH2 domain, but not the MH1 domain of Smad2 can be imported into nucleus in a cytosol independent manner [83]. Smad2 MH2 domain interacts with the phenylalanine-glycine

Smad	Import/export Factor	Derterminants on Smad proteins	Mechanisms R	eference
Nuclea Smad1	r import presumably Importin β	NLS like sequence in Smad1 MH1 do- main K ³⁹ KI KKKK ⁴⁵	Mutation of this NLS significantly impaired BMP induced nuclear lo- calization of GEP Smad1	[80]
Smad1	CAN/ Nup214	A conserved hydrophobic region on the MH2 domain	Phosphorylation in the linker region Px(S/T)P motifs leads to blockade of Smad1/Nup214 interaction and cyto plasmic retention of Smad1	n [46] of o-
Smad2	2 CAN/ Nup214	A "hydrophobic corridor" on the Smad2 MH2 domain	The conserved K ⁵⁰ KLKK ⁵⁴ sequence in Smad2 is not functional due to the insertion of exon 3; instead, Smad2 uses its MH2 domain to be imported	ce [84] le d
Smad3	Importin β & Ran	NLS like sequence on the MH1 domain K ⁴⁰ KLKK ⁴⁴	Smad3 binding to import β through its NLS is enhanced by TGF- β type I receptor mediated phosphorylation	gh [81, 82]
Smad3	CAN/ Nup214	A "hydrophobic corridor" on MH2 domain	In a nuclear import assay carried ou using digitonin permeabilized cells, Smad3 is imported in the absence of exogenously added cytosol	ıt [83] f
Smad4	Importin α	An extended bipartite basic NLS in the MH1 domain	Mutation of Smad4 bipartite NLS leads to loss of Smad4's transcriptic activity	[79] on
Smad4	CAN/ Nup214	Full length Smad4	monomeric Smad4 can also enter the nucleus in an alternative route throu nucleoporins other than CAN/Nup2	ne [83] 1gh 14
Nuclear export				
Smad1	CRM-1	Leucine-rich NESs In MH2 domain and MH1-linker junction	Smad1 export is inhibited by lepto- mycin B, a CRM-1 inhibitor; Smad NES is required for its transcription activity	[80] 1
Smad2	CAN/ Nup214 & Nup153	"Hydrophobic corri- dor" on the MH2 domain	Smad2 export does not require CRM-1; Smad2 export occurs in the absence of soluble factors	[84]
Smad3	Exportin 4 & Ran	aa 271-324 in MH2 domain	Smad3 export is CRM-1 independe	nt [86]
Smad4	CRM-1	Leucine-rich NES at MH1-linker junction	Smad4 export is abolished by lep- tomycin B, a CRM-1 inhibitor; Smad4-CRM-1 interaction is abolished by Smad3/Smad4 binding	[85]

Table 1.4 Smad nuclear import and export

Abbreviations: NLS, <u>N</u>uclear <u>L</u>ocalization <u>S</u>ignal; NES, <u>N</u>uclear <u>E</u>xport <u>S</u>ignal; CRM-1, <u>C</u>hromosome <u>Region M</u>aintenance-1; aa, <u>a</u>mino <u>a</u>cid residue

(FG)-repeats of the nucleoporins Nup214 and Nup153 directly via a surface "hydrophobic corridor". The MH2 region of Smad1 and Smad3 can also mediate nuclear import via a similar hydrophobic surface [84]. Interestingly, the Smad1-Nup214 interaction is prevented by Smurf1 binding to the Smad1 linker region [45]. The binding of Smurf1 in turn is triggered by linker Px(S/T)P motif phosphorylation by MAPKs. Therefore, MAPKs abolish Smad1 import by disrupting Smad1-Nup214 interaction through induction of Smurf1 binding. It remains to be determined whether MAPK can also inhibit Smad2/3 nuclear localization through a similar mechanism.

After nuclear translocation of the phospho-R-Smad-Smad4 complex, it does not stay there forever. Several observations support the notion that R-Smad and Smad4 can be exported from the nucleus [80, 85]. When the kinase activity of the TGF- β type I receptor is blocked by a specific inhibitor 60 minutes after ligand stimulation, Smad2, Smad3, and Smad4 that have already accumulated in the nucleus gradually distributes throughout nucleus and cytoplasm, accompanied by a decrease on C-tail phosphorylation. This suggests that: 1. Phospho-Smad2/3 are dephosphorylated in the nucleus by certain phosphatase(s); 2. After Smad2/3 dephosphorylation, significant portions of Smad2/3 and Smad4 translocate back into the cytoplasm. Wild type Smad1 are distributed both in the nucleus and in the cytoplasm in basal state. When two nuclear export signal (NES)-like sequences located at Smad1 MH1-linker junction and MH2 domain, respectively, are mutated, the resulting mutant Smad1 is located exclusively in the nucleus even without ligand stimulation. This suggests that the basal state distribution of Smad1 is the result of an equilibrium of nuclear import and export, and that blocking nuclear export leads to disruption of this equilibrium. Smad4 is present in

cytoplasm as well as nucleus in the absence of TGF- β superfamily ligands. Blocking the export factor chromosome region maintenance 1 (CRM-1) induces Smad4 nuclear accumulation. This indicates that Smad4 has intrinsic ability to enter and exit the nucleus, and that blocking CRM-1 dependent Smad4 export leads to the dominance of Smad4 nuclear import.

Smads export can occur through interacting with adaptor proteins (i.e. exportins) or via direct interaction with nucleoporins, which are NPC constituents. Smad1 contain two leucine-rich NESs (Nuclear Export Signals), one located at the MH1 domain-linker junction, and the other in the MH2 domain. Smad4 contain an NES in the N-terminal portion of the linker region. Both Smad1 and Smad4 are exported through their NES(s) in a CRM-1 dependent manner [78, 80, 85]. Since the main Smad1 NES is located right beside the linker region, it will be interesting to determine whether Smad1 linker phosphorylation has any effect on the function of this NES. Smad2 and Smad3 also contain a conserved NES-like sequence similar to the one in the Smad1 MH1-linker junction, but this NES is non-functional in Smad2 and Smad3. Smad2 undergoes nuclear export through direct binding to nucleoporins using a hydrophobic region of its MH2 domain, the same region that also mediates its import. In contrast, The MH2 domain of Smad3 interacts with exportin 4, which mediates its export in a Ran-dependent manner [86].

TGF- β superfamily ligands induce nuclear accumulation of Smad1, Smad2, Smad3, and Smad4. Nuclear accumulation of Smads can result from increased nuclear import, decreased nuclear export, or both. The mechanisms of ligand-stimulated nuclear accumulation of different Smads are distinct from each other. It has been speculated that

Smad1 C-terminal phosphorylation results in an increase in import and a decrease in export, and the latter may play a major role. This proposal remains to be tested. Smad2 is bound by SARA in the basal state, and this interaction excludes Smad2 interaction with Nup214. Upon ligand stimulation, SARA is dissociated from Smad2, allowing Smad2 to interact with nucleoporins and transport to nucleus. At the same time, phosphorylation of Smad2 increases its affinity for nuclear factors such as Forkhead Activin Signal Transducer (Fast-1). Interaction with Fast-1 blocks the export of nuclear Smad2. For Smad3, C-tail phosphorylation exposes an NLS in its MH1 domain and induces Smad3 to interact with importin- β . Interaction with importin- β promotes Smad3 nuclear translocation. Additionally, phosphorylated Smad3 has lower affinity for the export factor exportin 4, and a lower propensity to be exported. Since Smad4 is not directly phosphorylated by type I reeptor, the ligand dependent nuclear localization of Smad4 is regulated by the heteromerization with phosphorylated R-Smad. Formation of phospho-R-Smad-Smad4 complex has two functions in promoting Smad4 nuclear accumulation: first, R-Smad nuclear translocation will also bring the R-Smad bound Smad4 into the nucleus; Second, R-Smad interaction with Smad4 masks the Smad4 NES. It has been demonstrated that Smad3-Smad4 complex formation abolishes Smad4 binding of the export factor CRM-1 [87]. Thus, R-Smad bound Smad4 is unable to undergo nuclear export.

What is the function of Smad nuclear import and export? Since Smads are transcription factors, it is obvious that Smad import upon receptor activation is essential for its function in the nucleus. The NLS mutated Smad3 not only is defective in
activating TGF- β target genes but also dominant-negatively blocks TGF- β signaling. Mutation of Smad1 NLS severely impairs its transcription activity.

Nuclear export of Smad proteins is also an integral part of TGF- β ligands signaling. The exit of dephosphorylated Smad2/3 from the nucleus in the process of TGF- β signaling provides a mechanism by which intracellular Smad2/3 can continuously monitor the activity of type I receptor on the membrane [85]. After nuclear export of dephosphorylated Smad2 and Smad3, if T β RI is still active, Smad2/3 can be rephosphorylated, re-enter the nucleus, and continue to signal; if T β RI is not active any more, the dephosphorylated Smad2/3 will stay in the cytoplasm, returning to its basal state. When the NESs in Smad1 are mutated, the resulting Smad1 mutants have significantly lower activities towards reporter genes, although these mutants reside in the nucleus even without BMP treatment. This suggests that a portion of Smad1 nuclear localization impairs signaling activity.

In conclusion, Smad1, Smad2, Smad3, and Smad4 are all nucleus-cytoplasm shuttling proteins. Although different Smads enter and exit the nucleus by distinct mechanisms, the ability to shuttle is essential for the functioning of all R-Smads and co-Smad.

1.2.9 Transcriptional activity of Smads

1.2.9.1 DNA binding

R-Smad and Co-Smad are transcription factors. The MH1 domain of R-Smad (except Smad2) and Smad4 selectively bind to the palindromic sequence GTCTAGAC selected in a PCR-based enrichment process [34]. Structural studies of Smad3 MH1 domain complexed with its target sequence revealed that each MH1 domain binds to one GTCT sequence using a conserved β -hairpin structure [33]. The 'G' in the first position of GTCT and the "G" and "A" on the third and fourth positions of the opposite strand contact key amino acid side chains of MH1 domain through hydrogen bonds, while the second position "T" is not involved in hydrogen bond. This is consistent with the observation that the second position of GTCT (or third position of AGAC) can tolerate substitution without substantially decreasing binding affinity [34]. Smad2 MH1 domain cannot bind DNA because the insertion of exon 3 sequence disrupts the DNA binding hairpin structure. Independently, the sequence repeats of AGAC in the PAI-1 promoter was identified to be essential for Smad binding and TGF-β responsibility [88, 89]. In addition to the GTCT sequence, Smad can also bind GC rich motifs in the promoters of goosecoid, brinker, and vestigial [90-92].

1.2.9.2 Transcription partners

Smad binding sequence is too short to confer enough specificity and binding affinity. Smads cooperate with other transcription factors, which bind nearby sites, to regulate target genes (Table 1.5). A classic example of the cooperation between Smad and DNA binding partners is the ligand induced formation of the activin response factor (ARF) on the activin responsive element (ARE) of the *Xenopus* Mix.2 promoter. ARF contains three components: Smad2, Smad4 and Forkhead activin signal transducer (Fast) -1.

Smad binding Partners	Interacting Smad and domains	Classification of partners	Features/Mechanisms of action
ATF-2	Smad3/4 (MH1)	bZIP family	cooperate with Smad3/4 to activate CRE-luc and 3TP-Luc reporters
ATF-3	Smad3 (MH2)	bZIP family	cooperate with Smad3 to repress Id1 gene expression
c-fos	Smad3 (MH2) Smad4	bZIP family	cooperate with Smad3/4 for AP-1 dependent target gene expression
c-Jun, JunB, JunD	Smad3(MH1) Smad4	bZIP family	cooperate with Smad3/4 for AP-1 site transcription activation
E2F4/5	Smad2/3 (MH2)	bHLH	recruit p107 to Smad3 to repress c-myc expression
FoxH1/FAST	Smad2/3 (MH2)	forkhead family	formation of activin-response factor on Mix.2 promoter
FoxO	Smad3/4 (MH1)	forkhead family	cooperate to activate p21Cip1 and other target genes
GATA4,5,6	Smad1 (MH1)	Zinc finger family	cooperate with Smad1/4 to activate Smad7 and Nkx2 5 promoters
HIF-1α	Smad3 (MH1,2)	bHLH-PAS	cooperate with Smad3 to activate
Lef1/TCF	Smad3 (MH1,2) Smad2/4	HMG box	cooperate in activating the
Milk/Mixer	Smad2 (MH2)	homeodomain family	cooperate to activate <i>goosecoid</i> expression
OAZ	Smad1 (MH2)	Zinc finger family	cooperate to bind the BRE in and activate Xvent-2 promoter
p53	Smad2/3 (MH1)	p53 family	Cooperate to regulate a subset of TGF-ß target genes
Schnurri	Smad1 (MH1)	Zinc finger	regulate PPARv2, brinker genes
Sp1	Smad2 (MH2)	Zinc finger	cooperate to activate many genes
1	Smad3/4 (MH1)	family	e.g. p15Ink4B, p21Cip1, Smad7
SRF	Smad3	MADS-box family	mediate TGF- β activated SM22 α expression
TFE3	Smad3/4 (MH1)	bHLH	cooperate with Smad3/4 to activate PAI-1, Smad7 promoter
YY1	Smad1/4 (MH1)	Zinc finger family	function together with Smads, GATA to activate Nkx2.5

Table 1.5 Smad-interacting transcription factors

This table is adapted from reference [93]. Abbreviations: ATF, <u>Activating Transcription</u> <u>Factor</u>; FoxH1, <u>Forkhead box H1</u>; HIF, <u>Hypoxia Inducible Factor</u>; Lef1, <u>Lymphoid</u> <u>Enhancer-binding Factor 1</u>; TCF, <u>T Cell Factor</u>; OAZ, <u>Olf-1/EBF Associated Zinc</u> finger; SRF, <u>Serum Response Factor</u>; YY1, <u>Yin Yang-1</u> Upon activin treatment of cells, Smad2 MH2 domain binds a C-terminal region of Fast-1. Smad2 also binds Smad4 after activin stimulation, but Smad4 does not interact with Fast-1 directly. Therefore, Smad2 act as a bridging factor in the activin-induced formation of Smad2-Smad4-Fast-1 complex. In ARF, Fast-1 binds to AAATGT repeat sequences of ARE whereas Smad4 binds to GTCT sequence in the ARE [101, 102]. Other transcription factors can act in a similar fashion as Fast-1, by interacting with both specific DNA sequence and Smads to recruit Smad proteins to their low affinity binding sites. Many Smad interacting transcription factors are themselves under the control of other cellular signaling pathways (e.g. AP-1 is activated by c-Jun N-terminal kinase (JNK) pathway; FoxO is inhibited by PI3K pathway). The cooperation between these factors and Smads allows signal integration of TGF- β and other signaling pathways at the level of target promoters (Table 1.5).

1.2.9.3 Co-activators

Smad can recruit three classes of co-activators to regulate transcription (See Table 1.6 for a summary). The first class of co-activators are histone acetyl transferases (HATs) that can loosen up chromatin packaging by acetylating lysine residues on histone tails. Smad1/2/3/4 can recruit the HAT p300/CREB-binding protein (CBP) [36-41]. The interaction domains were mapped to a C-terminal fragment in p300 and the MH2 domain of Smads. The interaction of Smads with p300 is essential for their activity, and the sequestration of p300/CBP by adenovirus E1A protein abolishes transcriptional activation of Smads. Another two HATs, P/CAF and GCN5, interact with

Table 1.0	5	Smad	co-activators
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Co-activators	Interacting Smads and domains	Function/Mechanisms of action
p300 CBP	Smad1/2/3/4 (MH2)	important mediators of Smad transcription co-activation, histone acetyl transferases
	Smad3 linker	
P/CAF	Smad3 (MH2 and linker)	enhance Smad/p300 mediated transcription activation, belong to histone acetyl
	Smad2	transferases (HAT)
GCN5	Smad3 (MH2)	required for TGF- β /BMP signaling and
	Smad1/2/5	transcription activation, belong to HATs
MSG1/	Smad4 (MH2)	bind p300 and Smad4 at the same time
Cited1		recruit p300 to Smad4
$\delta EF1/ZEB-1$	Smad1/2/3	recruit p300/CBP and/or P/CAF
		to Smads
Cited2/	Smad2/3	promote p300/Smad mediated
MRG1	(MH2)	transcription
SRC-1	no direct inter-	interact with p300, potentiate p300/
	action with Smads	Smad3 interaction and transcription
ARC105	Smad2/4 (MH2)	required for TGF-β/activin/nodal
	Smad3	signaling in Xenopus and cells
SWI/SNF ATP-	Smad2/3/4	required for TGF- β /Smad target
ase Brg1	(MH2)	gene activation

This table is adapted from reference [93]. Abbreviations: P/CAF, <u>p300/CBP</u> <u>A</u>ssociated <u>Factor</u>; CBP, <u>CRE-binding protein Binding Protein</u>; MSG1, <u>Melanocyte-Specific Gene</u> 1; Cited, <u>CBP/p300-interacting transactivators with glutamic acid (<u>E</u>)/aspartic acid (<u>D</u>)-rich C-terminal domain; MRG1, <u>Melanocyte-specific gene 1 (MSG1)-Related Gene 1</u>; SRC-1, <u>Steroid Receptor Co-actvator-1</u>; ARC105, <u>Activator-Recruited Co-factor 105</u>; SWI, <u>SWI</u>TCH; SNF, <u>Sucrose Non Fermenting</u>; Brg1, <u>B</u>rahma-<u>Related Gene 1</u></u>

MH2 domain of Smad2/3 and potentiate TGF- β signaling [35, 103]. GCN5 can additionally bind BMP specific R-Smads (Smad1/5).

In addition to MH2 domain, another region in Smad4 linker region (aa 276-322) that is rich in proline is also required for transcription activation [37]. This region, termed Smad Activation Domain (SAD), interacts with the EVH1 domain of the co-activator SMIF [104, 105]. The Smad3 linker region, which is rich in proline but bears little homology with Smad4 SAD, also contains a transcription activation domain [106, 107]. The Smad3 linker region interacts with p300 and P/CAF.

The second class of co-activator is the ARC105/Mediator complex [108]. The mediator is a multisubunit complex of 20-30 proteins. It bridges specific transcriptional activators with general transcriptional machinery. Smad2/3 and Smad4 can interact specifically with the ARC105/Med15 subunit of the mediator, and depletion of ARC105 by RNA interference results in suppression of TGF- β /activin/Nodal signaling in mouse. The observations highlight the essential role of mediator in Smad mediated recruitment of basic transcription machinery.

In response to TGF- β stimulation, Smad2, 3 and 4 interacts with Brg1, the ATPase subunit of the chromatin remodeling complex SWI/SNF, at the endogenous level [109]. Depletion of Brg1 from nuclear extracts inhibited phospho-Smad2 mediated *in vitro* transcription of target genes. Furthermore, Brg1 associates with the promoter of the TGF- β target gene *lefty1*, as revealed by chromatin immunoprecipitation. Small interfering RNA (siRNA) mediated Brg1 knockdown significantly decreases activin induced expression of endogenous *lefty1*, and to a lesser extent, *Nodal*. These findings indicate that the SWI/SNF chromatin remodeling complex is essential for the expression of at least a subset of TGF- β /activin responsive genes.

In summary, three distinct classes of co-activators, histone acetyl transferases, chromatin remodeling complex, and the mediator, can be recruited to target promoters by activated Smad complexes. These three classes of co-activators work through very different mechanisms to activate transcription. Histone acetyl transferases (HAT), such as p300, P/CAF, and GCN5, put acetyl groups to lysine residues of the N-terminal flexible tail of core histones. This modification can directly reduce the histone-DNA interaction, or alternatively, recruit some other factors to make the histone-DNA complex (the chromatin) less tight and more accessible. Chromatin remodeling complexes such as SWI/SNF do not chemically modify histone. Instead, they alter the physical structure of chromatin by hydrolyzing ATP. The mediator complex, on the contrary, does not influence chromatin structure but directly recruit the RNA polymerase II enzyme complex.

What is the interplay of different co-activators in mediating Smad activated target gene transcription? In an *in vitro* transcription system, phospho-Smad only activates transcription from chromatin template [109]. In contrast, several other transcription factors such as p53 can mediate transcription activation on naked DNA. The ability of most transcription factors to activate transcription on naked DNA template reflects the direct assembly of RNA pol II holoenzyme on promoter DNA by these transcription factors. Although Smads can also recruit mediator complex, which can recruit RNA pol II, it is not sufficient for Smad transcription activation. In vitro transcription is inhibited by p300 HAT inhibitor, or the depletion of Brg1 from nuclear extract, suggesting both

p300 and Brg1 are required for transcription activation by Smads. These findings are consistent with a two-step model of transcription activation by Smad: first, activated Smad complex recruits chromatin modifying enzymes such as p300 and the SWI/SNF chromatin remodeling complex. This converts tightly-packaged chromatin template to a more accessible state; in the second step, the mediator complex recruits the RNA polymerase II to engage in transcription.

1.2.9.4 Co-repressors

Histone Deacetylase (HDAC) and Histone Methyl Trasferase (HMT) modify specific lysines of histone tails by removing acetyl group and adding methyl group, respectively. The modification will change the way the nucleosomes are packaged, or result in the recruitment of heterochromatin specific protein HP1, eventually leading to a compact, repressed chromatin structure. Smad1/5, but not Smad 2/3, directly associates with the HMT Suv39h [110]. The Smad3 MH1 domain is associated with HDAC activity, but the interaction may not be direct [111]. The MH2 domain of Smad2/3 recruits the repressors Ski, SnoN, and TGIF [112, 113] which themselves can recruit HDAC activities. Some of Smad co-repressors are summarized in Table 1.7.

Ski and SnoN are related proteins. Ski represses Smad signaling by multiple mechanisms, but mainly through recruitment of HDAC, and the simultaneous inhibition of p300 recruitment by Smads [114]. Ski overexpression leads to cell transformation by abolishing the TGF- β growth inhibition, as seen in melanoma [112].

TGIF recruits mSin3 and CtBP to Smad2/3; mSin3 and CtBP in turn recruits HDAC activities [115, 116]. TGIF competes with p300/CBP for binding Smad2/3. The balance

Co-repressors	Interacting Smads and domains	Features/Mechanisms of action
Ski	Smad2/3/4 (MH2)	recruit HDAC complexes NcoR and mSin3, inhibit Smad/p300 interaction
SnoN	Smad2/3/4 (MH2)	recruit HDAC activities? Induced by TGF-β and BMP
TGIF	Smad1/2/3 (MH2)	recruit CtBP and mSin3 to Smads, CtBP mSin3 are HDAC containing complexes
HDAC	Smad3 (MH1) Indirect	HDAC activities were found to indirectly associate with Smad3 MH1 domain
SIP1/ δEF-2	Smad1/2/3/5 (MH2)	recruit CtBP to target promoters
SNIP1	Smad1/2 Smad4 (MH1,2)	inhibit the formation of Smad4/p300 transcriptional complex
Evi-1	Smad(MH2) Smad1/2/4	repress TGF-β/Smad3 transcription by recruiting CtBP
Dach1	Smad1/4	recruit NcoR and mSin3a to repress BMP specific Smads

Table 1.7 Smad co-repressors

This table is adapted from reference [93]. Abbreviations: SnoN, <u>Ski-related Novel gene</u> <u>N</u>; TGIF, <u>TG</u> Interacting <u>Factor</u>; HDAC, <u>Histone DeAcetylase</u>; SIP1, <u>Smad Interacting</u> <u>Protein 1</u>; SNIP1, <u>Smad Nuclear Interacting Protein 1</u>; Evi-1, <u>Ecotropic virus integration</u> site-1; Dach1, <u>Dachshund 1</u>; CtBP, <u>C-terminal Binding Protein</u>; mSin3a, <u>mammalian</u> homolog of yeast <u>SWI-independent 3A</u>; NcoR, <u>Nuclear hormone receptor <u>co-Repressor</u></u>

between the mutually exclusive Smad-TGIF and the Smad-p300 complexes is very important for cellular function [113]. The loss of one copy of TGIF gene causes the devastating genetic disease holoprosencephaly.

1.3 Dephosphorylation of Smad C-terminal domain

The phosphorylation of the C-tail of receptor-regulated Smads (R-Smads) is the "on" switch of the entire TGF- β /Smad signaling pathway, resulting in the accumulation of R-Smad and Smad4 in the nucleus, where they regulate gene transcription in accompany with other factors. Smad signaling is eventually "switched off" in the nucleus. Although Smad signaling can be irreversibly terminated by Smad degradation, the major portion of phospho-Smad is dephosphorylated in the nucleus. Dephosphorylation leads to the dissociation of R-Smad from Smad4. Dephosphorylated R-Smads have lower affinity for other transcription factors, co-activators, and co-repressors. Dephosphorylation of R-Smads also restores their affinity for cytoplasmic retention factors (e.g. SARA for Smad2/3). Un-phosphorylated R-Smads have higher rate of nuclear export and lower rate of nuclear import. Therefore, dephosphorylation of R-Smads terminates their nuclear function and transports them back into the cytoplasm. The dephosphorylation of R-Smad has at least two functions in TGF- β /smad signaling. The first function is that dephosphorylation of R-Smads allows them to be recycled back into cytoplasm, where they can monitor the activity of the membrane type I receptor. If the type I receptor is still active, they will be re-phosphorylated and re-enter the nucleus. The second function is that when the type I receptor is no longer active, the dephosphorylation of R-Smads

allows them to go back to the cytoplasm and stay there, assuming their basal state again [85].

The observation that a major part of phosphorylated R-Smads are dephosphorylated and returned to the cytoplasm after the blockade of type I receptor activity predicts the existence of phosphatases for R-Smads. Since C-terminal motif of R-Smads are phosphorylated on serines, potential candidates of C-tail phosphoatases include protein serine/threenine phosphatases (P(S/T)P) which dephosphorylate phospho-serine or phospho-threonine, and dual-specific phosphhatases (DUSP) which cleave the phosphates on serine, threenine as well as tyrosine. In the mammalian genome, there are three families of protein serine/three phosphatases (P(S/T)P): the PPM family, the PPP (phosphoprotein phosphatase) family, and the FCP1 (TFIIF-associated Carboxylterminal domain Phosphatase)/SCP (Small pol II Carboxyl-terminal domain Phosphatases) family [99]. Among these, the PPM family and FCP1/SCP family phosphatases are metal-ion dependent phosphatases. Although there are considerably less protein phosphatases than kinases, phosphatases can interact with distinct regulatory subunits to target different substrates. Recently, several phosphatases have been identified that dephosphorylate phosphorylated R-Smad C-tail (Table 1.8, [94-99]).

In the search for the phosphatases that dephosphorylate the TGF- β specific R-Smad Smad2/3, Lin et al created expression plasmids for the catalytic subunits for 39 P(S/T)Ps and DUSPs [99]. When individual phosphatases are co-expressed with Flag-tagged Smad2/3 together with constitutively active TGF- β type I receptor T β RI (T202D) in human embryonic kidney cells 293T (HEK 293T cells), only PPM1A/PP2C α significantly reduced the phosphorylation of Flag-Smad2/3 induced by T β RI (T202D).

Phosphatase	Substrate	Identification method
PPM1A	Phosphorylated Smad2/3 C-tail	Screening of 39 phosphatases for the phosphatases that decrease the C-tail phosphorylation of Smad2 and Smad3 co-expressed w/ caTβRI in 293 cells
PPM1A	Phosphorylated Smad1/5/8 C-tail	Assumption based on the fact that Smad1/5/8 have similar C-tail structure with Smad2/3, and that PPM1A dephosphorylates phosphorylated Smad2/3 C-tail, which was tested and proved by experiment.
PDP	Phosphorylated Mad/Smad1 C-tail	Screening 44 protein S/T phosphatases by RNAi in S2 cells looking for the phosphatases whose knockdown leads to delayed dephosphory- lation of Mad C-tail domain after Dpp removal
SCP1/2/3	Phosphorylated Smad1 C-tail	An unbiased expression cloning screen for factors that affect <i>Xenopus</i> Dorsal/Ventral polarity identi- fied Xenopus Os4, a homologue of mammalian SCP2. Further experiments showed that Smad1 C- tail can be dephosphorylated by all three SCPs.
SCP1/2/3	Phosphorylated Smad1/2 linker	This was assumed based on the fact that SCP1/2/3 physically interact with Smad1 and Smad2. The assumption was verified by experiments.
SCP1/2/3	Phosphorylated Smad2/3 linker & MH1 domain	Screening of 41 phosphatases for phosphatases that decrease/abolish the phosphorylation of Smad2/3 linker (S/T)-P motifs identified SCP1/2/3

To be continued

Table 1.8 continued

Phosphatase (Substrate)	Cellular Localization	Interac In vitre	ction with substrate o In vivo	Dephosphory In vitro	lation In vivo
PPM1A (Smad2/3 C-tail)	predominantly in nucleus	Yes	Yes, in 293T and HaCaT cells	Yes	Yes
PPM1A (Smad1/5/8 C-tail)	same as above	Yes	Yes, in 293T cells	Yes	Yes
PDP (Mad/Smad1 C-tail)	nucleus, cytoplasm mitochondria	Yes	Yes, in S2 cells	Yes	N.D.
SCP1/2/3 (Smad1 C-tail)	predominantly in nucleus	N.D.	Yes, in 293 cells	Yes	Yes
SCP1/2/3 (Smad1/2 linker)	same as above	N.D.	Yes, in 293 cells	Yes	Yes
SCP1/2/3 (Smad2/3 linker, N domain)	same as above	Yes	Yes, in 293T cells	Yes	Yes

To be continued

Phosphatase (Substrate)	Effect of Depletion	Effect of Over-expression	Reference
PPM1A (Smad2/3 C-tail)	Inceased TGF-β stimulated Smad2/3 C-tail phosphory- lation, increased Smad2/3 nuclear accumulation and Smad2/3-Smad4 association Increased expression of reporter and endogenous genes, increased sensitivity of cells to TGF-β mediated growth inhibition	Strongly decreased Smad2/3 C-tail phosphorylation and increased Smad2 export blunted response of TGF-β target genes, decreased sen- sitivity of cells to TGF-β medi- ated growth inhibition	[99]
PPM1A (Smad1/5/8 C-tail)	Increased BMP-2 induced Smad1/5/8 phosphorylation on C-tail SxS motif, and Increased expression of p21 and Id1 reporter genes and endogenous genes, both the basal level and BMP-2 sti- mulated level	Strongly decreased/abolished BMP-2 induced Smad1/5/8 C- tail phosphorylation; Decreased Smad1/4 interaction; Decreased GAL4-Smad1, GCCG, Id1, and Xvent2-luc reporter genes both with and without BMP-2 treat- ment.	[95]
PDP (Mad/Smad1 C-tail)	In Drosophila S2 cells and in mammalian cells, RNAi mediated depletion of PDPs caused significant delay of the disappearance of p-Mad/ Smad1 after receptor activity was blocked. The activities of the reporters 2xUbx-LacZ and Smad6-luc increased	The activity of the Drosophila reporter gene 2XUbx-LacZ was dose dependently inhibited by PDP overexpression	[97]
SCP1/2/3 (Smad1 C-tail)	RNAi inhibition of SCPs enhances BMP-stimulated Smad1 C-tail phosphoryla tion and delays the decrease of C-tail phosphorylation with time. SCP RNAi en- hances expression of endo- genous BMP target genes: Id1, Smad6, SnoN	SCP1/2/3 individually over- expressed can strongly inhibit the BMP responsive reporter gene BRE-luc	[96]

To be continue

Phosphatase (Substrate)	Effects of Depletion	Effects of Over-expression	Reference
SCP1/2/3 (Smad1/2 linker)	Increased activity of Smad1 toward Id1,decreased Smad2 /Smad3 activity on several targets including Smad7,p15 INK4B, p21Cip1 and Notch signaling ligand JAGGED1	Decreased phosphorylation of Smad1 and Smad2 linker (S/T)- motifs	[94] .P
SCP1/2/3 (Smad2/3 linker & N domain)	Increased phosphorylation on the (S/T)-P motifs in the linker region of Smad2/3	Strongly decreased/abolished Smad2/3 linker and MH1 do- main (S/T)-P motifs phosphory- lation; Increased activities of the TGF- β responsive reporter genes SBE-luc, p15-luc and p21-luc; reversed the inhibition of p15 expression by EGF	[98]

Abbreviations or explanations: PDP, Pyruvate Dehydrogenase Phosphatase; SCP, Small RNA polymerase II Carboxyl-terminal domain (CTD) Phosphoatase; RNAi, RNA interference; MH1 domain, Mad Homology 1 domain; (S/T)-P motif, serine/threonine-proline motif, potential sites for proline directed protein kinases; N.D., Not Determined; p21, CDK inhibitor p21WAF1/Cip1; Id1, Inhibitor of Differentiation 1; p-Mad, phosphorylated Mad (on the C-tail); Ubx, Ultrabithorax; BRE, BMP Response Element; SnoN, Ski-related novel gene N; p15, the CDK inhibitor p15INK4B; SBE, Smad Binding Element; EGF, Epidermal Growth Factor

PPM1A/PP2C α is a member of the PPM family of Mg²⁺-requiring protein phosphatases. In an in vitro binding assay, PPM1A strongly prefers to bind phosphorylated Smad2 MH2 domain. PPM1A also interacts with Smad2 at the endogenous level in a TGF-β dependent manner. Recombinant PPM1A can dephosphorylate semi-synthetic phospho-Smad2 MH2 domain (p-S2MH2). Knock-down of PPM1A with RNA interference (RNAi) delayed the time-dependent decrease in Smad2/3 C-terminal phosphorylation after receptor blockade by the kinase inhibitor SB431542. This is consistent with the role of PPM1A in removing C-tail phosphorylation in TGF-β signaling. Potentiated Smad2/3 C-tail phosphorylation brings about several consequences: in PPM1A knock-down cells, TGF- β induced a more robust nuclear accumulation of Smad2 and Smad3 and more Smad2-Smad4 and Smad3-Smad4 association was observed. The prolonged Smad signaling in HaCaT cells stably expressing small hairpin RNA targeting PPM1A (shPPM1A) leads to a global enhancement of TGF- β signaling. TGF- β induced expression of the CDK inhibitor p15 and p21, the extracellular matrix regulator plasminogen activator inhibitor (PAI-1) and fibronectin (FN), was more pronounced in shPPM1A expression cells. On the other hand, TGF- β repressed c-myc mRNA to a lower level in PPM1A knock-down cells than in control cells. Consistent with the stronger response of growth-related genes p15, p21, and c-myc to TGF- β treatment in PPM1A shPNA expressing cells, the TGF- β induced growth arrest was more complete in shPPM1A HaCaT cells.

In contrast to PPM1A knock-down, the overexpression of PPM1A attenuated TGF- β signaling. Co-expression of PPM1A decreased the interaction between Smad2/3 and Smad4. In a highly-sensitive nuclear export assay, the expression of wild type PPM1A,

but not the catalytically-inactive D239N mutant, markedly increased the Smad2-export mediated expression of a reporter gene. These observations suggest that PPM1A promotes the dephosphorylation of C-tail phosphorylated Smad2/3, leading to their dissociation from Smad4 and subsequent nuclear export. Expression of PPM1A in zebra fish embryos phenocopied the disruption of signaling of *Nodal*, a homolog of mammalian TGF- β . In addition, co-expression of PPM1A with Smad2 in zebra fish embryos strongly suppressed the dorsalizing effect of Smad2.

Interestingly, the depletion of PPM1A from cells by RNAi not only increased TGF- β stimulated expression of p15 and p21, but also increased the basal level of p15 and p21 mRNA. This reflects that the antocrine mediated activation of TGF- β /Smad signaling contributes to the expression of target genes in the absence of exogenous TGF- β , and that endogenous PPM1A represses the basal state expression of p15 and p21 by dephosphorylating the C-tail phosphorylated Smad2/3 resulting from autocrine TGF- β signaling.

In similar over-expression experiments in 293T cells, PPM1A is also able to dephosphorylate the BMP specific Smad1, 5 and 8 [95]. Unlike Smad2/3, which is only dephosphorylated by PPM1A among the 39 phosphatases tested, Smad1/5/8 can be dephosphorylated by several other phosphatases in addition to PPM1A when they are over-expressed. The ability of PPM1A to reduce the phosphorylation of Smad1 C-tail induced by constitutively active BMP type I receptor (ALK3 Q233D) ruled out the possibility that PPM1A indirectly inhibit Smad1 phosphorylation by targeting BMP type I receptor. Interestingly, PPM1A directly interacts with both the N- and the C-domain of GST-Smad1 in *in vitro* binding assay. An interaction between PPM1A and Smad1 was

also detected *in vivo* when both proteins were co-expressed in 293T cells. When PPM1A was over-expressed, the BMP2 induced activation of GAL4-Smad1, GCCG-Luc, Id1-Luc, and Xvent2-luc reporter genes was abolished. On the contrary, when endogenous PPM1A was knocked down, the activity of BMP target reporter genes Id1-Luc and p21-Luc was enhanced both in the absence and in the presence of BMP2. The induction of endogenous p21 and Id1 mRNA by BMP2 was also enhanced. These augmented gene responses were accompanied by an increase of BMP2 induced Smad1 C-terminal phosphorylation.

PPM1A localizes to the nucleus of cells. R-Smads (Smad1, 5, 8; Smad2, 3), when phosphorylated at the C-tail, also accumulate in the nucleus together with Smad4. The nuclear distribution of PPM1A is consistent with its role in dephosphorylating the activated R-Smad complexes in the nucleus.

Besides the newly identified Smad2/3 C-tail, PPM1A also acts on and dephosphorylates phosphorylated p38, CDK2, phosphotidylinositol 3-kinase (PI3K), and axin. Notably, p38, PI3K, and Glycogen Synthase Kinase 3 (GSK3), a downstream target of PI3K, are all activated by TGF- β in certain contexts. However, the inhibitory effect of PPM1A overexpression on TGF- β signaling is mediated by Smads, but not by these non-Smad targets.

In contrast to the over-expression based screen to identify Smad2/3 C-tail phosphatases, an RNAi based (loss of function) functional genomic screen was conducted to search for phosphatases that affect the phosphorylation status of Mad, the *Drosophila* homologue of Smad1 [97]. It was found that among the 44 protein phosphatases examined, only the knock-down of pyruvate dehydrogenase phosphotase

(PDP) led to significant retention of phosphorylated Mad (p-Mad) after removal of Dpp (the *Drosophila* BMP homologue). PDP belongs to the PPM family of protein phosphatases whose catalytic domain is similar to that of PP2C. PDP is a maternal effect gene in dephosphorylating and inhibiting Mad, and loss of only one copy of the gene from the mother is sufficient to lead to ectopic expression of p-Mad in the embryos. PDP associates with Mad both in the basal state and upon Dpp stimulation in *Drosophila* S2 cells, but the interaction seemed stronger in the presence of Dpp. In addition, both the cytoplasmic and the nuclear localized PDP interact with Smad1. There are two PDPs in mammals, PDP1 and PDP2. Knock down of both PDPs led to enhanced BMP4stimulated Smad1 C-tail phosphorylation and delayed dephosphorylation in Hela cells, whereas single knock down only had partial effect. In contrast, depletion of PDPs does not lead to enhanced phosphorylation of Smad2/3, suggesting that PDP is specific for the BMP pathway Smads.

Pyruvate dehydrogenase phosphatase (PDP) is a phosphatase implicated in metabolic regulation. Besides Mad/Smad1, the metabolic enzyme pyruvate dehydrogense is the only known substrate of PDP to date. PDP localizes to mitochondria, cytoplasm, and nucleus. It is not known whether only the nuclear pool of PDP dephosphorylate p-Smad1, or PDP from all three locations (nucleus, cytoplasm, mitochondria) can dephosphorylate Smad1 in certain circumstances.

In an unbiased screen to identify modifiers of development of *Xenopus* dorsal/ventral polarity, the *Xenopus* homologue of human Os4 (XOs4), was shown to induce a second axis when over-expressed in *Xenopus* embryos [96]. Induction of a second axis could result from either the inhibition of BMP pathway or activation of the activin pathway.

Careful examination of the expression of BMP and activin target genes in XOs4 overexpressing Xenopus ectodermal explant revealed that XOs4 expression inhibits the BMP/Smad1 pathway, rather than activates the activin pathway. Human Os4, also known as Small RNA polymerase II Carboxyl-terminal domain Phosphatase 2 (SCP2), belong to a family of three protein phosphatases SCP1, SCP2, and SCP3, which are homologous to the catalytic domain of TFIIF associated Carboxyl-terminal domain Phosphotase 1 (FCP1). These two lines of evidence, namely, the fact that SCP2 inhibits BMP signaling, and that SCP2 is a protein phosphatase, immediately raises the possibility that SCP2 may inhibit BMP signaling by dephosphorylating Smad1 on the SxS motif. Biochemical analysis demonstrated that SCPs bind Smad1 in a BMPindependent manner, and that bacterially expressed Xenopus SCP2 (XSCP2) can dephosphorylate Smad1 in vitro. Depletion of SCP1/2 by RNAi increased BMP4 induced Smad1 C-tail phosphorylation. Consequently, the BMP responsive reporter gene BRE-Luc showed more robust activation by BMP in the presence of SCP2 small interfering RNA (siRNA). In addition, the BMP-stimulated endogenous Id1, Smad6, and SnoN expression was much stronger when SCP2 was depleted.

SCPs are nuclear phosphatases. Their localization fits their role as a phosphatase of the C-tail of Smad1, which accumulates in the nucleus after its C-terminal SxS motif is phosphorylated by BMP stimulation. Another phosphatase, FCP1 which is homologous to SCPs, dephosphorylates the RNA polymerase II (pol II) carboxyl-terminal domain (CTD) phosphorylated on serine 2 and serine 5. Given the critical role played by CTD in regulating the functional status of pol II, FCP1 is an important regulator of the RNA polymerase II transcription cycle. Since SCPs can also dephosphorylate CTD in vitro, SCPs are potential regulators of pol II mediated transcription. The identification of SCPs as Smad1 phosphatase may suggest a potential link between Smad1 and the pol II trascription machinery.

In summary, PPM1A is the only phosphatase demonstrated to date to dephosphorylate the C-terminal domain of Smad2 and Smad3. However, several phosphatases, including PPM1A, SCPs, and PDPs can dephosphorylate the C-terminal domain of BMP specific Smad1. Knock-down of any one of the above phosphatases failed to completely block the time-dependent decrerase in Smad1 phosphorylation after removal of BMP. Therefore, multiple phosphatases may function redundantly to dephosphorylate Smad1 C-tail SxS motif.

R-Smads have highly conserved C-tail sequence. Indeed, PPM1A recognizes and dephosphorylates the phospho-C-tail of both the TGF- β /activin specific Smad2/3 and the BMP specific Smad1/5/8. However, SCPs and PDPs discriminate between R-Smads of the TGF- β branch and the BMP branch. SCP1/2/3 and PDP1/2 show strong activity to dephosphorylate the phosphorylated C-tail of Smad1, whereas they can only slightly, if at all, dephosphotylate phospho-C-tail of Smad2/3. Thus, PPM1A has broader substrate specificity among R-Smads than SCPs and PDPs.

The activity and specificity of many phosphatases are regulated by their interaction with various regulatory subunits. In mammalian cells, depletion of SCP or PDP (catalytic subunits) leads to increased Smad1 C-tail phosphorylation, suggesting that SCP and PDP are necessary for Smad1 C-tail dephosphorylation under physiological conditions. However, overexpression of these phosphatases in 293T cells together with Smad1 failed to repress Smad1 C-terminal phosphorylation, suggesting that SCP and PDP are not sufficient to dephosphoryate Smad1, and other factors may be required. On the contrary, PPM1A alone when over-expressed, can dephosphorylate the C-tail of all R-Smads. Thus, PPM1A functions as Smad phosphatases independent of additional regulatory subunits. Interestingly, SCP1/2/3 can dephosphorylate Smad1 when overexpressed in *Xenopus* embryos, indicating that Xenopus embryos, but not 293T cells, may contain sufficient amounts of the cofactor required for SCP to dephosphorylate Smad1 C-tail.

The amount of phosphorylated C-terminal SxS motif of R-Smads is the main determinant of signaling strength of TGF- β or BMP. The phosphorylation of R-Smad Ctail is jointly controlled by activated type I receptor and C-tail phosphatases. When PPM1A, SCP, or PDP are depleted by RNAi, a delayed C-tail dephosphorylation as well as a global increase of ligand stimulated activities of both reporter genes and endogenous genes were observed. On the contrary, overexpression of PPM1A or SCP abolished the ligand induced activation of TGF- β or BMP target promoters, accompanied by a dramatic loss of ligand induced C-tail phosphorylation. Therefore, C-tail phosphatases are potent negative regulators of TGF- β /BMP signaling, influencing both the strength and the duration of signaling. Interestingly, depletion of the phosphatases also increased basal activities of certain promoters targeted by TGF- β or BMP pathways. This suggests that in the resting state (i.e. in the absence of ligand), the C-tail phosphatases may serve to dephosphorylate aberrant C-tail activation by autocrine signaling. In summary, C-tail phosphatases represses basal signaling, and controls the strength and duration of ligand stimulated signaling of TGF- β ligands.

Although PPM1A, SCP, and PDP are crucial regulators of TGF- β ligands signaling, their subcellular localization does not seem to be affected by TGF- β /BMP treatment. In addition, TGF- β does not seem to influence the expression or phosphatase activity of PPM1A. Thus, Smad C-tail phosphatases are not implicated in negative feedback regulatory loops to control Smad signaling.

PPM1A, SCP, and PDP can all interact with their substrates in the absence of ligand treatment. The interaction of endogenous Smad2 and PPM1A was significantly increased upon TGF- β stimulation; phosphorylated Smad2 MH2 domain has much higher affinity for PPM1A. Similarly, Dpp pathway activation increases Mad-PDP interaction by several folds. In contrast, the interaction of SCP with Smad1 and Smad2 is not influenced by constitutively active receptor.

Of the three phosphatases, PPM1A and SCP are predominantly nuclear, whereas PDP localizes to nucleus, cytoplasm, and the mitochondria. Phosphorylation induced nuclear accumulation of Smads upon ligand stimulation leads to colocalization of Smads with PPM1A and SCP in the nucleus. However, BMP induced Smad1/Mad nuclear translocation does not seem to favor the colocalization of Smad1/Mad with PDP, which is predominantly cytoplasmic.

Smad activation may lead to its accelerated dephosphorylation. This is not achieved by a change of the localization or expression of the phosphatases. Rather, Smad C-tail phosphorylation changes localization of Smads and/or their affinity for the phosphatases. TGF-β treatment can increase the rate of Smad2 dephosphorylation both by colocalization of Smad2 with PPM1A and by an increase of its intrinsic affinity for PPM1A. Smad1 C-tail phosphorylation does not increase its affinity for SCP, but its nuclear translocation leads to colocalization with SCP, promoting its dephosphorylation by SCP. For Mad/Smad1 activated by BMP, their nuclear translocation does not result in their co-distribution with PDP, but their activation increases their intrinsic affinity for PDP, facilitating their dephosphorylation by PDP.

Although dephosphorylation of R-Smad C-terminal SSxS motif represents the main mechanism of the disappearance of phosphorylated R-Smads, the proteasome mediated degradation also contributes to the time-dependent clearance of phospho-Smad. Indeed, treatment of cells with the proteasome inhibitor MG132 slowed down the disappearance of phospho-Smad2 and phospho-Smad1 after the blockade of their upstream signaling. Therefore, receptor kinases, multiple phosphatases, and proteasome pathways cooperate with each other in the initiation and termination of Smad signaling.

1.4 Proteasome mediated Smad degradation

Dephosphorylation reversibly represses Smad signaling by converting phosphorylated Smad into un-phosphorylated Smad, decreasing the pool of phospho-Smad while increasing the pool of un-phosphorylated Smad. Dephosphorylation allows the phospho-Smad to be "switched off" and recycled. In contrast, degradation of Smad irreversibly terminates TGF- β signaling. Proteasome mediated Smad turnover is not limited to phosphorylated Smad. Un-phosphorylated R-Smad and the Co-Smad Smad4 can also be targeted by the proteasome pathway. In addition, other proteins of the TGF- β pathway, such as the type I receptor and the co-repressor SnoN, also undergo regulated destruction through the proteasome pathway. Thus, the ubiquitin-proteasome system represents a more versatile regulator of TGF- β /Smad signaling (Table 1.9).

Target protein Smads	Ubiqutin E3 ligase	Adaptor Protein	Explanation/Mechanisms
Smad1/5	Smurf1	none	Smurf1 inhibits lung branching morphogenesis and osteogenesis, promotes myogenesis
Smad1/5	Smurf1	Smad6	Smad1/5 (Δ PY) can be degraded via Smad6
Smad1/4	CHIP	none	CHIP inhibit Smad1/4 mediated signaling
Smad1	Smurf2	none	Smurf2 targets Smad1/2, but not Smad3
Smad1	none	HsN3/ Az	Smad1 directly interacts w/ proteasome subunit HsN3 and ornithine decarboxylase antizyme Az to be degraded by the proteasome
Smad2	Smurf2	none	Smurf2 targets Smad2 at higher concentration than Smad1_activated Smad2 are preferred
Smad2	WWP1	TGIF	TGIF itself is not degraded in the process
Smad2	Nedd4-2	none	WWP1 and Nedd4-2 are HECT E3 ligases
Smad2	Itch/AIP4	none	Ubiquitination does not result in degragation
			Rather, it promote Smad2 C-tail phos- phorylation
Smad3	$SCF^{\beta TrCP1}$	none	Activated Smad3 interact with SCF component
			Roc1 to be targeted to the ubiquitin-proteasome
Smad3	CHIP	none	CHIP mediated Smad3 degradation is inde- pendent of ligand stimulation, inhibits TGF-β
			signaling
Smad4	$SCF^{\beta TrCP1}$	Jab1	Jab1 enhances $SCF^{\beta TrCP1}$ mediated Smad4
Smad4	SCF ^{Skp2}	none	SCF ^{Skp2} induces degradation of cancer derived Smad4 mutants
Smad4	Smurf1/2	Smad2 Smad6 Smad7	HECT domain E3 ligases Smurf1/2, Nedd4-2
	Nedd4-2	Smad7	WWP1/Tiul1 through their interaction with the
	WWP1	Smad7	PY motifs in I-Smads and R-Smads
Medea	dSCF ^{βTrCP1}	?	only genetic evidence available, Slimb is the <i>Drosophila</i> homologue of βTrCP1
Medea	Highwire	none	only genetic evidence is available
Smad4	ectodermin	none	genetic evidence and biochemical evidence
Smad6	Smurf1/2	none	Smad6 act as adaptors for Smad4, type I
			receptors, and Smad1/5, and degraded at the same time in complex with those proteins.
Smad7	Smurf1/2	none	Smad7 is degraded together when it acts as adaptor protein for TGF-β type I receptor and Smad4 turnover

Table 1.9 Ubiquitination mediated degradation of TGF- β signaling components and related proteins

Continued on next page

Table 1.9 Continued

Target protein	Ubiqutin E3 ligase	Adaptor protein	Explanation/Mechanisms
Smad7	Arkadia	none	Arkadia augment TGF-β signaling, the balance between acetylation by p300 and deacetylation by Sirt1 and HDAC influences Smad7 stability
TODI	Smarrf1/2	Smad7	Smad7 recruite Smurf1/2 to degrade TRDI
Тркі	Sillui 11/2	Sinad /	Sinad/ recruits Sinur1/2 to degrade TPRI
BMP RI	Smurfl	Smad6	Smad6 recruits Smurf1 to degrade BMP RI
Co-repressors	5		
SnoN	Smurf2 APC	Smad2/3	MH2 domain and linker PY motif of Smad2/3 recruits APC and Smurf2 respectively
SNIP1	none	Smad1	Smad1 interacts with proteasome subunit HsN3 and Az to directly recruit SNIP1 to proteasome
other proteins	,		n n i i n i i j i i n i i i i r
HEF1	APC	Smad3	TGF- β treatment induces HEF1 degradation
Cdc25A	$\mathrm{AIP4/Itch}$ $\mathrm{SCF}^{\beta\mathrm{TrCP1}}$	Smad3	APC does not play a role in Smad3 induced
Runx2	Smurf1	Smad6	Smurf1 can also directly interact and induce the degradation of Runx2
β-catenin	Smurf2	Smad7	Smad7 recruits Smurf2 to β-catenin

This table is based on reference [100], Abbreviations or explanations: Smurf, <u>Smad</u> <u>ubiquitination regulatory factor;</u> CHIP, <u>C</u>arboxyl terminus of <u>H</u>sc70-Interacting <u>P</u>rotein; HsN3, a b-type proteasome subunit homologous to PRE4 from yeast, X1 b from Xenopus and RN3 from the rat; Az, ornithine decacrboxylase antizyme; WWP1, <u>WW</u> domain-containing <u>P</u>rotein 1; Tiul1, <u>T</u>GIF interacting <u>ubiquitin ligase 1; TGIF, <u>TG</u> Interacting <u>Factor;</u> Nedd4-2, <u>N</u>euronal precursor cell-<u>expressed developmentally downregulated 4-2; HECT, <u>H</u>omologous to <u>E</u>6 associated protein <u>C</u>arboxyl <u>T</u>erminus; Itch, product of the mouse <u>Itchy</u> gene locus; AIP4, <u>A</u>trophin 1-Interacting <u>P</u>rotein 4; SCF, <u>Skp1-Cullin-F</u>-box protein complex of E3 ubiquitin ligase; β TrCP1, <u> β -T</u>ransducin <u>repeat</u> <u>Containing Protein 1; Jab1, Jun activation domain-binding protein 1; SnoN, Ski-related</u> <u>novel gene N; SNIP1, Smad Nuclear Interacting Protein 1; HEF1, <u>H</u>uman <u>E</u>nhancer of <u>Filamentation 1; APC, Anaphase-Promoting Complex; Runx2, Runt homology domain</u> transcription factor 2</u></u></u>

A protein named Smad ubiquitination regulatory factor 1 (Smurf1) was identified in a yeast two-hybrid screen using *Xenopus* Smad1 as the bait [117]. Smurf1 bears significant homology to the Hect subclass of E3 ubiquitin ligases. Smurfl contains an Nterminal calcium-binding C2 domain, two WW domains that can bind PPxY motifs in partner proteins, and a carboxyl-terminal Hect domain which is involved in ubiquitin transfer reaction. In 293T cells and COS-1 cells, Smurf1 overexpression specifically decreases the steady state level of co-transfected Smad1 or Smad5 protein, but does not significantly affect the level of Smad2, 3 or 4. A catalytic inactive mutant of Smurfl (C710A) binds both Smad1 and Smad5, but does not associate with Smad2. Thus, Smurfl is a BMP pathway specific ubiquitin E3 ligase. In Xenopus embryos, the overexpression of Smurfl in ventral marginal zone (VMZ) dorsalizes the embryos and triggers the ectopic formation of an axial. Smurf1 mRNA injection also antagonizes the ventralization effect of Smad1 overexpression in *Xenopus* embryos. In conclusion, Smurf1 selectively targets the BMP specific R-Smad Smad1/5 for proteasome mediated degradation, and inhibits the activity of the BMP pathway.

The interaction of Smurf1 and Smad1 requires the PPAY motif in the linker region of Smad1. Deletion of the PPAY residues from the Smad1 sequence abolishes its interaction with Smurf1. Consequently, the deletion of PPAY motif results in the resistance of the mutant Smad1 (Δ PY) to Smurf1 mediated degradation [117]. The Smad1 linker region contains four Px(S/T)P motifs that are consensus sites for mitogen-activated protein kinase (MAPK). An intriguing finding is that the MAPK mediated Px(S/T)P phosphorylation is required for Smad1 to bind Smurf1 [45]. The binding of Smurf1 to Smad1 can have two distinct results: First, Smurf1 binding to Smad1 may

leads to Smad1 degradation by the proteasome; Second, Smurf1 binding blocks the binding of the nucleoporin CAN/Nup214, and inhibits the nuclear accumulation of Smad1. These two effects of Smurf1 on Smad1, namely proteasome mediated degradation and nuclear exclusion, are not necessarily mutually exclusive. They may be two independent mechanisms of Smurf1 inhibition of Smad1 signaling. Therefore, Smurf1 can inhibit Smad1 signaling without involving the ubiquitin-proteasome pathway. The dependence of Smad1-Smurf1 association on Smad1 linker phosphorylation connects together two important regulators of Smad1 function: the MAPK pathway and the ubiquitin ligase Smurf1.

The ubiquitin E3 ligases that control Smad1 stability is not limited to WW domain containing ubiquitin ligase Smurf1. CHIP (carboxyl-terminus of Hsc70 interacting protein), a U-box dependent E3 ubiquitin ligase, interacts with the MH2 domain of Smad1. The steady state level of Smad1 and Smad4 was significantly reduced by the overexpression of CHIP, whereas the proteasome inhibitor MG132 prevented the CHIP mediated Smad1/4 degradation [118]. Expression of CHIP inhibits the Smad1/4 induced activation of the BMP specific reporter (GCCG)₁₂-Luc; on the contrary, RNA interference of CHIP increases the Smad1/4 mediated transcription activity.

In a genome-wide search for Smurf1 homologues, the protein Smurf2 was identified [119, 120]. Smurf2 is 83% identical in amino acid sequence with Smurf1 and has a similar domain structure with Smurf1: an N-terminal C2 domain, two WW domains, and a C-terminal Hect domain. Although Smurf2 binds to Smad1, Smad2 and Smad3, Smurf2 only mediates the ubiquitination and degradation of Smad1 and Smad2, but not that of Smad3. Similar with Smurf1, the association of Smurf2 with Smads also depends

on the presence of the PPxY motifs in the linker region of R-Smads. It is interesting that Smad2 and Smad3 also contains several (S/T)-P motifs located in the vincinity of the PPxY motifs in their linker regions. (S/T)-P motifs are potential sites for proline-directed protein kinases, such as MAPKs, cyclin-dependent kinases (CDKs), and Glycogen Synthase Kinase-3 (GSK3). Since the phosphorylation of Px(S/T)P motifs in Smad1 linker region is required for its interaction with Smurf1, it is interesting to ask whether the phosphorylation on the Smad2/3 linker region also affects their interaction with Smurf2.

Although Smad3 associates with Smurf2, its degradation is not induced by Smurf2 overexpression [119, 120]. Instead, Smad3 assocaites with the Ring finger protein Roc1 through its MH2 domain in a TGF- β dependent manner [121]. Roc1 is a subunit of the SCF (Skp1, Cullin, and F-box protein) ubiquitin ligase complex. SCF complexes are ubiquitin ligases with fixed Roc1, Skp1 and Cullin subunits and a variable F-box protein. Smad3, through its interaction with Roc1, associates with the SCF E3 ligase $SCF^{\beta TrCP1}$ containing Roc1, Skp1, Cullin and the F-box protein Fbw1a/βTrCP1 (β-Transducin repeats Containing Protein 1). Interestingly, the binding of Smad3 to the co-activator p300 promotes the interaction between Smad3 and $SCF^{\beta TrCP1}$. Once bound by the $SCF^{\beta TrCP1}$ ubiquitin ligase, Smad3 is exported to the cytoplasm for degradation. Since the SCF^{β TrCP1} and Smad3 interaction is enhanced by TGF- β stimulation, the SCF^{β TrCP1} ubiquitin ligase mainly targets the activated Smad3. A U-box dependent E3 ligase, CHIP, interacts with Smad3 and induces its degradation. CHIP mediated Smad3 degradation is independent of TGF- β stimulation [122]. Stable expression of CHIP in Mv1Lu (a mink lung epithelial cell line that is highly responsive to TGF- β) cells reduced Smad3 level and significantly decreased the sensitivity of cells to TGF- β mediated gene activation. Thus, unlike SCF^{β TrCP1}, which degrades phosphorylated Smad3, the ubiquitin ligase CHIP controls the Smad3 level in the basal state and regulates the competence of cells to respond to TGF- β .

Smad4 does not contain a PPxY motif as in R-Smads and I-Smads, but Smad4 can also be targeted by WW domain containing E3 ligases such as Smurf1/2 through its interaction with R-Smads and I-Smads [123]. In this case, the R-Smads and I-Smads act as adaptor proteins that bring together Smad4 and its E3 ligase Smurf1/2.

Smad4 interacts with the F-box protein β -TrCP1, and through binding β -TrCP1 Smad4 associates with the SCF ubiquitin ligase $SCF^{\beta TrCP1}$ [124, 126]. Expression of $SCF^{\beta TrCP1}$ components reduced the half-life of co-transfected Smad4, whereas the expression of β -TrCP1 siRNA increased the level of endogenous Smad4. Therefore, the ubiquitin ligase $SCF^{\beta TrCP1}$ controls the turnover and the steady state protein level of endogenous Smad4. The expression of Jab1 (Jun activation domain binding protein 1), a component of the COP9 signalosome, increases the interaction between Smad4 and β-TrCP1, thereby facilitating Smad4 degradation through the proteasome pathway [127]. Consistent with the effect of Jab1 on Smad4- $SCF^{\beta TrCP1}$ interaction, co-expression of Jab1 with SCF components jointly reduces the transcription activity of Smad4. Interestingly, the F-box protein Skp2 also interacts with Smad4 in a protein array based screening. The E3 ligase SCF^{Skp2} interacts much stronger with the cancer-derived, unstable Smad4 mutant R100T than with wild type Smad4, and induces its degradation [125]. Thus, wild type Smad4 and cancer-derived mutant R100T may be targeted to the proteasome pathway through different SCF ubiquitin ligases.

Interestingly, Highwire, a putative RING finger ontaining E3 ubiqitin ligase, represses a signaling cascade at the *Drosophila* neuralmuscular synapses involving Wit, Tkv, Sax, Mad, and Medea [128]. This pathway is a *Drosophila* BMP-like pathway influencing the development of the *Drodophila* neural muscular junction structure (NMJ). *Drosophila* that is deficient in Highwire shows enlarged neural-muscle junction (NMJ) structure, a result of overactivation of the synaptic BMP signaling. Highwire binds to the *Drosophila* Co-Smad Medea directly, but it is not known whether the repression of Mad signaling by Highwire is through ubiquitin-proteasome dependent or independent mechanisms.

Another RING type E3 ubiquitin ligase, Ectodermin, was identified in a functioinal screen for *Xenopus* ectoderm determinants [129]. Ectodermin is a maternal determinant of *Xenopus* ectoderm. Ectodermin directly binds and ubiquitinates Smad4. Since Smad4 is essential for both BMP and TGF- β signaling, Ectodermin inhibits the signal transduction of both BMP and TGF- β pathways. In Xenopus embryonic development, Ectodermin inhibits TGF- β like signals, and antagonizes the mesoderm inducing activity of TGF- β /activin. Thus, the presence of Ectodermin inhibits mesoderm differentiation and promotes ectoderm development. Interestingly, the knock down of endogenous Ectodermin attenuates the degradation of cancer derived, unstable Smad4 mutant R100T. Consequently, RNAi targeting Ectodermin sensitizes the cells to TGF- β 's cytostatic effects. In conclusion, Ectodermin may be an important regulator both in embryonic development and tumorigenesis through its function as a Smad4 E3 ligase.

In summary, Smad4 can be targeted by three classes of E3 ubiquitin ligases, namely WW domain and HECT domain containing E3 ligases (e.g. Smurf1, Smurf2), SCF

ubiquitin E3 ligases (e.g. $SCF^{\beta TRcP1/2}$, and SCF^{Skp2}), and Ring finger type E3 ligases (e.g. Highwire, Ectodermin). Control of Smad4 stability has important implication both in embryonic development and in cancer development.

Smad proteins are not only the targets of ubiquitin mediated degradation, they can also act as adaptor molecules in proteasome mediated turnover of the co-repressor SnoN [52] and the TGF- β type I receptor [55]. Smad2 binds to Smurf2 through its PPGY motif in the linker region, and Smad2 also associates with the Smad co-repressor SnoN. Thus, Smad2 binding to both Smurf2 and SnoN brings together the two proteins into a Smurf2-Smad2-SnoN ternary complex. In this complex, SnoN is subsequently ubiquitinated by Smurf2 and targeted to the proteasome. Interestingly, Smad2 itself is not very efficiently ubiquitinated by Smurf2. The Smurf2-Smad2 interaction is more likely involved in the degradation of SnoN, rather than the turnover of Smad2 itself. Deletion of the PPGY motif in Smad2 linker region abolishes the ability of Smad2 to mediate SnoN degradation. Notably, Threonine 220 of the Smad2 linker region, a putative site for proline-directedprotein kinase, is located right before the PPGY motif (T²²⁰PPPGY). In addition, three (S/T)-P sites (S245, S250, S255) reside in the vicinity of the PPGY motif in the Smad2 linker region. All four proline-directed kinase sites, T220, S245, S250, S255, are phosphorylated in certain circumstances. It is not known how the phosphorylation on these sites influences the binding of Smurf2.

Smad3, which also bind Smurf2, can play a similar role in recruiting Smurf2 to SnoN [52]. In addition, Smad3 associates with CDH1, an activator of the multi-protein ubiquitin ligase APC (anaphase promoting complex) via its MH2 domain [130]. Smad3 promotes the formation of a quarternary complex containing Smad3, SnoN, APC, and

CDH1. In this manner, Smad3 recruits APC to degrade SnoN through its interaction with both proteins.

Smad7, an inhibitory Smad, contains a PPxY motif in its linker region. Smad7 stably interact with the TGF- β type I receptor, and Smad7 interacts with Smurf2 using its PPxY motif. Upon activation of TGF- β signaling, Smad7 expression is induced. Smad7 then moves to the cytoplasm and brings Smurf2 to type I receptor. A complex of Smad7, Smurf2, and TGF- β receptors then forms on the plasma membrane, in which both Smad7 and TGF- β receptors are ubiquitinated and targeted to the proteasome pathway. Smad7 mediated T β RI degradation is a nagative feedback mechanism of TGF- β signaling control.

CDC25A is a phosphatase that positively regulates cell cycle progression. CDC25A promotes G1 and M phase progression by removing the inhibitory phosphorylation from CDK2 and CDK1. It was demonstrated that activation of the TGF- β pathway either by TGF- β treatment or overexpression of Smad3 dramatically downregulates the protein level of CDC25A [131]. The SCF type ubiquitin ligase SCF^{\betaTrCP1/2} and the multisubunit E3 ligase APC interact with CDC25A and mediate its proteasomal degradation. The interaction between CDC25A and SCF^{\betaTrCP1/2} is mediated by the association of CDC25A and the F-box proteins β TrCP1 or β TrCP2, which are the variable subunits of the E3 ligase SCF^{\betaTrCP1/2}. Interestingly, the association of CDC25A with β TrCP requires the presence of endogenous Smad3, and knockdown of endogenous Smad3 severely impairs the CDC25A- β TrCP interaction. In addition, Smad3 interacts with CDC25A in a TGF- β dependent manner, and the MH2 domain of Smad3 and Smad3 MH2 domain can

induce significant decrease in the protein level of co-transfected CDC25A. On the contrary, RNAi mediated Smad3 knockdown abolishes TGF- β induced CDC25A degradation. Thus, TGF- β induced, Smad3 mediated CDC25A degradation by the proteasome represents a novel mechanism by which TGF- β inhibits cell cycle progression.

In summary, R-Smads and Co-Smad interacts with different ubiquitin E3 ligases. The association of Smads and ubiquitin E3 ligases may have several consequences. First, binding of Smads by components of E3 ligases can lead to their proteasome mediated degradation. Smad1 interacts with Smurf1/2 through its PPxY motif (i.e. PY motif) in the linker region; Smad1 also associates with the U-box E3 ligase CHIP via its MH2 domain. Both Smurf1 and CHIP target Smad1 to the proteasome. Smad2 and Smad3 interact with Smurf2 through the PY motif in their linker region. But Smurf2 only moderately promotes Smad2 degradation, and does not affect Smad3 level. Instead, Smad3 interacts with the Roc1 subunit of the SCF ubiquitin ligase $SCF^{\beta TrCP1}$ via its MH2 domain, and $SCF^{\beta TrCP1}$, rather than Smurf2, leads to Smad3 degradation. Smad4 interacts with multiple E3 ubiquitin ligases, resulting in its degradation. Interaction of Smad4 with the F-box protein Skp2 was mapped to its MH1 domain, whereas Medea (the *Drosophila* Smad4) interacts with the Ring type E3 ligase Highwire (Hiw) through the MH2 domain.

The second consequence of Smad-E3 ligase interaction is the degradation of Smad interacting proteins. Smad2 and Smad3 interacts with Smurf2, but Smurf2 does not lead to efficient degradation of Smad2 or Smad3. Thus, interaction of Smad with E3 ligase does not necessarily lead to Smad degradation. The TGF- β induced association of

Smad2/3 with Smurf2 results in the degradation of the Smad-interacting co-repressor SnoN upon TGF- β stimulation. In addition, the MH2 domain of Smad2/3 interacts with the cell cycle related ubiquitin ligase APC, and recruits APC to degrade SnoN. Therefore, Smad2 and Smad3 recruit two kinds of ubiquitin E3 ligases, Smurf2 and APC, to induce SnoN degradation in a TGF- β dependent manner.

The third consequence of Smad binding by Smurf is nuclear exclusion. Smad1 binds Smurfl only when it is phosphorylated on the four Px(S/T)P motifs in the linker region. The binding of Smurf1 to the Smad1 linker region sterically blocks the binding of nucleoporin CAN/Nup214 to a hydrophobic corridor in its MH2 domain. Since the interaction of MH2 domain of Smads is essential for Smad nuclear translocation, the binding of Smurf1 to Smad1 linker region inhibits Smad1 nuclear localization by excluding nucleoporin binding to Smad1 MH2 domain. It is surprising that Smad1-Smurf1 binding requires both the PY motif and the phosphorylation on Px(S/T)P motifs. Since Smad2 and Smad3 also contain 4 (S/T)-P motifs in the linker region, it is interesting to ask whether phosphorylation on these (S/T)-P motifs is also required for Smad2/3-Smurf2 association. The hydrophobic surface ("hydrophobic corridor") on Smad2/3 not only binds nucleoporins; it is a versatile protein interaction motif that also binds SARA and nuclear co-factors such as Fast-1. Therefore, it is possible that Smurf binding to Smad2/3 may influence multiple aspects of Smad2/3 function, including receptor-mediated phosphorylation, nuclear translocation, and nuclear transcription function. However, this speculation has never been tested.

1.5 Regulation of Smad activity through phosphorylation by proline-directed kinases

1.5.1 Cyclin-Dependent Kinases (CDKs)

Cyclin dependent kinases play key roles in orchestrating cell cycle progression. G1 cell cycle progression malfunction is directly linked with cancer. G1 phase transition is governed by three CDKs, CDK2, CDK4 and CDK6. CDK2 binds to E type cyclins (cyclin E1, E2) and A type cyclins (cyclin A1, A2), whereas CDK4 and CDK6 associate with D type cyclins (cyclin D1, D2, D3). G1 CDKs, namely CDK2, 4, and 6, promote cell cycle progression by phosphorylating and inactivating Rb (Retinoblastoma) protein. Since Rb is a transcription repressor of genes that is required for cell cycle, its inactivation leads to the expression of cell cycle genes. In early G1, CDK4/6-cyclin D complexes phosphorylate Rb, and relieve the inhibition of Rb on cell cycle promoting genes. In mid-to-late G1 and S phase, the CDK2-cyclin E/A complexes further phosphorylate Rb, abolishing the inhibitory effects of Rb on cell cycle genes. It is generally considered that CDK4/6 initiates Rb phosphorylation, while CDK2 completes Rb hyper-phosphorylation.

Smad2/3 can be directly phosphorylated by all three G1 CDKs, CDK4, CDK6, and CDK2 in *in vitro* kinase assay [50]. Notably, besides Smad3, Rb is the only demonstrated substrate for CDK4. Mutation of all five (S/T)-P motifs in Smad3 linker region and MH1 domain (T8, T179, S204, S208, S213) abolished *in vitro* phosphorylation of Smad3 by CDKs. This suggests that CDK phosphorylation of Smad3 occurs within these five sites. Among these five putative CDK phosphorylation sites, three are Px(S/T)P motifs (PFT⁸P, PET¹⁷⁹P, and PMS²¹³P) and two are xx(S/T)P motifs
(S²⁰⁴P, S²⁰⁸P). Three of these sites, T179, S204, and S208, are phosphorylation sites for ERK/MAPK, and can be induced by EGF treatment.

Metabolic labeling of endogenous Smad3 with ³²P in Mv1Lu cells from different stages of cell cycle revealed that S phase cells incorporate the most radioactivity. The peak of ³²P incorporation into Smad3 coincides with the peak of CDK activity, strongly supporting the hypothesis that endogenous CDKs phosphorylate endogenous Smad3.

To map the sites in Smad3 that are phosphorylated *in vivo* (i.e., in cells), phosphospecific antibodies against all five potential sites were raised. Mv1Lu cells were synchronized in G0/G1 phase by contact inhibition and then split into fresh medium to allow cell cycle progression. Parallel plates were then harvested at dfferent time points after release from contact inhibition. These plates represent different stages of cell cycle. When the profiles of phosphorylation of different (S/T)-P motifs (T8, T179, S204, S208, and S213) were monitored by site-specific phospho-peptide antibodies, the phosphorylation of T8 and S213 matches the profile of CDK activity (peaks 8~11 hours after cells were split), whereas S204, and S208 phosphorylation peak coincides with that of ERK/MAPK activity (peaks at 1.5~3 hour after cells were split). The phosphorylation wave on T179 site during cell cycle progression reflects both the activity of ERK and that of CDK2/4. That is to say, T179 phosphorylation was high both at the $1.5 \sim 3$ hour time interval when ERK activity was high, and at 8~11 hours time interval when CDK activity was high. Judged by the sizes of the peaks of T179 phosphorylation, the extent of T179 phosphorylation by CDK was greater than its phosphorylation by ERK. In conclusion, three out of the five (S/T)-P motifs, T8, T179, and S213, are targeted by G1 CDKs, whereas a distinct set of (S/T)-P motifs, T179, S204, and S208, are MAPK sites

under physiological conditions (see Figure 1.5 for a summary). CDK and ERK/MAPK phosphorylation converges on site T179. Interestingly, all three CDK target sites in Smad3 are Px(S/T)P motifs.

To study the effects of CDK phosphorylation on Smad3 activity, Smad3 mutants in which the CDK target sites are mutated singly or in combination were used. Mutation of T8, T179, and/or S213 increases the activity of Smad3 towards the reporter gene p15-Luc as well as the endogenous p15 promoter. p15 is a CDK inhibitor that can bind to CDK4/6 and inhibit their kinase activity. In addition, mutation of CDK target sites of Smad3 increases its potency to inhibit both c-myc reporter and endogenous c-myc gene. c-myc is a growth-promoting gene. Therefore, mutation of CDK phosphorylation sites in Smad3 promotes Smad3 anti-proliferative activity characterized by more robust p15 induction and more potent c-myc repression. Indeed, CDK phosphorylation sites mutant Smad3 have more potent growth inhibitory effect when infected into primary MEF cells, as revealed by ³H-thymidine incorporation assay and direct cell number counting.

What are the mechanisms that CDK phosphorylation inhibits Smad3 antiproliferative activity? Since linker phosphorylation of Smad2/3 leads to their nuclear exclusion in certain cellular contexts (see 1.5.2, Smad phosphorylation by ERK/MAPK), and two of the CDK sites locate in the linker region, CDK phosphorylation may result in the inhibition of Smad3 nuclear accumulation. Alternatively, CDK phosphorylation can affect other aspects of Smad signaling. For example, we observed modest increase of the interaction between Smad3 and p300 when CDK target sites are mutated. To date, the mechanisms by which CDK phosphorylation represses Smad3 transcription activity have not been systematically investigated, and remain an open question.

Smad3 E Smad2 E	Smad3 P Smad2 P	Smad3 F Smad2 Y	Smad3 Smad2	Smad3 - Smad2 N	Smad3 L Smad2 L	Smad3 Smad2 G	Smad3 M Smad2 M	
 ▲ I I 2 ▲ I I 2 ▲ Q S N - I P E T P P G Y L S E D G E T S D H Q M N H S 199 ▲ Q S N Y I P E T P P P G Y I S E D G E T S D Q Q L N Q S 240 	th T E I P A E F P L D D Y S H S I P E N T N F P A G I 170 th T E I L T E L P P L D D Y T H S I P E N T N F P A G I 210 th T T T T L T E L T E L T T T T T T T T T	V F N M K K D E V C V N P Y H Y Q R V E T P V L P P V L V 140 V F N L K K D E V C V N P Y H Y Q R V E T P V L P P V L V 180	3 L Р Н V Т Y С - Р V <mark>R W Р D L Н S Н Н Е L R A M E</mark> L С Е 110 3 L Р Н V Т Y С R L W <mark>R W Р D L Н S Н Н Е L К A I E</mark> N С Е 150	IDQWDTTGLYSFSEQT RSLDGRLQVSHR 80 RSLDGRLQVSHR 120	5 KAITTQNV <mark>NTKCITIP</mark>	QNGQEEKWCEKAVKSLVKKLKKTGQLDE 3 QNGQEEKWCEKAVKSLVKKLKKTGRLDE 60	SILPFTPPIVKRLLGWKK SILPFTPPVVKRLLGWKKSAGGSGGAGG 30	T8
KVLTQMGSPSIRCSSVS KVLTQMGSPSVRCSSMS 467	G W G A E Y R R Q T V T S T P C W I E L H L N G P L Q W L D G W G A E Y R R Q T V T S T P C W I E L H L N G P L Q W L D 49	A A L L A Q S V N Q G F E A V Y Q L T R M C T I R M S F V K A A L L A Q S V N Q G F E A V Y Q L T R M C T I R M S F V K 4	N С N Q R Y G W H P А T V С К I P P G C N L К I F N N Q E F 34 N С N Q R Y G W H P А T V С К I P P G C N L К I F N N Q E F 35	H I G R G V R L Y Y I G G E V F A E C L S D S A I F V Q S P H I G R G V R L Y Y I G G E V F A E C L S D S A I F V Q S P 3	GFTDPSNSERFCLGLLSNVNRNAAVELTRR 26 GFTDPSNSERFCLGLLSNVNRNATVEMTRR 36	PAFWCSISYVELNQRVGETFHASQPSMTVD 25 PAFWCSIAYVELNQRVGETFHASQPSLTVD 30	MDAGSP-NLSPNPMSPAHNNLDLQPVTYCE MDTGSPAELSPTTLSPVNHSLDLQPVTYSE 22	S204 S208 S213

Figure 1.5 Summary of Smad3 and Smad2 phosphorylation by CDK and ERK MAP kinase. The linker regions of Smad3 and Smad2 are marked by brackets. For Smad3, it is amino acids 143-231. For Smad2, it is amino acids 183-273. In Smad3, T8, T179 and S213 are phosphorylated by CDK4 and CDK2 (dots). S208, S204 and T179 in Smad3 are phosphorylated by ERK MAP kinase in response to EGF stimulation (triangles).

Extracellular Signal Regulated Kinase (ERK)s belong to the Mitogen Activated Protein Kinase (MAPK) superfamily. ERK1/2 are essential components of a signaling cascade of three kinases, the MAPK kinase kinase (MAPKKK) Raf, the MAPK kinases (MAPKKs) MEK1/2, and the MAPKs ERK1/2. This pathway is activated by growth factors that signal through receptor tyrosine kinases. By phosphorylating downstream targets such as transcription factors (e.g. Elk-1) or other protein serine/threonine kinases, ERK1/2 convert growth factor signals into changes in target gene expression, or changes in non-transcriptional processes. In several studies, Smad1, Smad2 and Smad3 were found to be substrates of ERK/MAPK [45-49]. The results are discussed in detail below.

Smad1 is a phosphoprotein even in the absence of BMP signals [47]. The basal phosphorylation of Smad1 is significantly increased upon treatment of cells by EGF or HGF, two activators of the ERK MAPK. Mutation of the C-terminal SSVS motif did not abolish basal Smad1 phosphorylation. This suggests that ERK may phosphorylate Smad1 on sites that is different from the C-terminal SSxS sites. ERK2 phosphorylates recombinant Smad1 in vitro. Edman degradation and mass spectrometry of Smad1 in vitro phosphorylated by ERK2 located phosphorylation sites to four PxSP motifs, namely, PHS¹⁸⁷P, PNS¹⁹⁵P, PHS²⁰⁶P, PGS²¹⁴P. To map ERK sites in vivo, Smad1 overexpressed in R1B/L17 cells (a Mv1Lu-derived cell line which is deficient in TGF-β type I receptor) and labeled with ³²P was subjected to protease digestion and resolved on a gel. The size of radioactive bands indicated that the phosphorylation sites reside in the linker region. To precisely map the phosphorylation sites, groups of serines or threonines

in the Smad1 linker region were mutated. Only the mutation of the serines in PxSP motifs affected the labeling of Smad1 by ³²P. Thus, both in cells and *in vitro*, Smad1 is phosphorylated by ERK on the four PxSP motifs in the linker region. Smad1 is also phosphorylated in the linker region when overexpressed in NIH3T3 cells [45], *Xenopus* whole embryos, *Xenopus* animal caps, and *Xenopus* oocytes upon FGF8 (Fibroblast Growth Factor 8) or IGF2 (Insulin-like Growth Factor 2) treatment [46], as indicated by a slightly larger molecular weight on SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). A Smad1 mutated on the serines of the four PxSP motifs does not show the change in molecular weight upon growth factor treatment [46]. In addition, endogenous Smad1 in NIH 3T3 cells is phosphorylated when cells are treated with FGF8, as indicated by the band position change caused by a slightly larger molecular weight on SDS-PAGE. Wild type Smad1, but not the linker mutant Smad1, is phosphorylated in Xenopus embryos without exogenously added growth factors. This suggests that linker region phosphorylation occurs in response to endogenous signals.

The effects of linker phosphorylation on Smad1 signaling have been examined either by treating cells with the MEK inhibitor U0126 or by mutating the phosphorylation sites. Smad1 EPSM (ERK/Proline-directed kinase Sites Mutant), in which the four PxSP motifs are mutated to PxAP, demonstrated significant nuclear localization when overexpressed in COS-1 cells even in the absence of BMP [47]. The wild type Smad1 overexpressed in COS-1 cells is distributed both in the cytoplasm and nucleus. When COS-1 cells overexpressing wild type Smad1 were treated with U0126 (MEK inhibitor), the wild type Smad1 was significantly shifted to nuclear distribution, resembling the distribution of mutant Smad1. When cells were treated with BMP, wild type Smad1 accumulated in the nucleus of the cell. But when the cells were treated with EGF (an ERK activator) at the same time, the BMP induced Smad1 nuclear translocation was blocked. Taken together, these results suggested that both in the basal state and in the presence of BMP, ERK phosphorylation inhibits Smad1 nuclear localization.

The effects of Smad1 linker phosphorylation on its subcellular distribution were examined in a more physiological setting in Smad1 linker mutant knock-in mice [132]. In these mice, the wild type Smad1 locus was replaced by a Smad1 mutated on the four PxSP motifs and one GSK3 site. In these mice (Smad1^{L/L}), the mutant Smad1 protein was expressed at endogenous levels. In gastric parietal cells from wild type mice, Smad1 was distributed uniformally in cytoplasm and nucleus, whereas in parietal cells from Smad1^{L/L} mice, Smad1 cleared the cytoplasm but accumulated in the nuleus and also localized to the cell membrane. Overall this is consistent with the results that overexpressed Smad1 EPSM mutant has a more nuclear localization. The distribution of mutant Smad1 to the membrane was an interesting observation. The biological significance of this phenomenon is not completely understood. Treatment of MEFs (Mouse Embryonic Fibroblasts) with MEK inhibitor U0126 also results in the localization of Smad1 to the nucleus as well as the plasma membrane. These observations indicated that phosphorylation of the endogenous Smad1 linker region prevented its retention on plasma membrane and at the same time also inhibited its nuclear accumulation.

When both mutant Smad1 and wild type Smad1 were overexpressed in cell lines or in *Xenopus* embryos, they have very similar steady-state protein levels. This seems to suggest that Smad1 linker phosphorylation does not affect their protein stability and turnover [47]. But overexression of Smad1 may saturate endogenous factors and mask the effect of linker phosphorylation. Treatment of MEFs (Mouse Embryonic Fibroblasts) from wild type mice, but not those from Smad1^{L/L} mice by FGF modestly decreased the protein level of Smad1 [45]. This suggests that although linker phosphorylation of Smad1 did not change their overexpressed levels, it does affect endogenous Smad1 protein level. Linker mutated Smad1 was resistant to phosphorylation-induced degradation of Smad1.

Smad1 linker phosphorylation downregulates its activity by at least two means: inhibition of nuclear translocation, and promotion of proteasome-mediated degradation. A uniformed mechanism has been discovered for the two phenomena. Linker phosphorylation of Smad1 induced its binding to the ubiquitin E3 ligase Smurf1. The association of Smad1 and Smurf1 is mediated by the WW domains in Smurf1 and the PPAY motif in the Smad1 linker region. Since the ERK phosphorylation sites PxSP motifs are located in the vincinity of the PPAY motif that is essential for Smurf1 binding, the phosphorylation status of PxSP motifs may influence Smurf1 binding. This notion is supported by the observation that only GST-Smad1 pre-phosphorylated in the linker region by ERK, but not the un-phosphorylated recombinant protein GST-Smad1, bound to overexpressed Smurfl in vitro. In addition, the requirement for nearby phospho-residues by the Smurf1 WW domains to bind to PPxY motifs is a unique feature that has not been described previously for Smurf1 WW domains. The Smad1 linker region is at the bottom of a hydrophobic surface in the MH2 domain that mediates interaction of R-Smad with nucleoporins, cytoplasmic anchoring protein and nuclear transcriptional partners. Thus, the binding of Smurf1 to Smad1 linker region may affect binding of other partner proteins to Smad1. Indeed, pre-incubation of linker phosphorylated Smad1 with Smurf1 blocked the subsequent binding of the nucleoporin CAN/Nup214 to Smad1. Taken together, linker phosphorylation of Smad1 induces Smurf1 binding, and at the same time inhibits nucleoporin binding. Thus, Smad1 linker phosphorylation leads to two consequences: the ubiquitination and degradation of Smad1, and the blocking of nuclear translocation.

At the cellular level, Smad1 linker phosphorylation inhibits its nuclear accumulation. To further study Smad1 linker phosphorylation using a reporter gene as a readout, both the wild type Smad1 (Smad1WT) and the linker mutant Smad1 (Smad1EPSM) were cloned into the GAL4 DNA binding domain fusion vector [47]. Both GAL4-Smad1 (WT) and GAL4-Smad1 (EPSM) respond to BMP treatment by increasing transcription activity several folds. When treated with EGF at the same time, the induction fold of wild type Smad1 was dramatically decreased, whereas the induction of Smad1 EPSM was not affected. Thus, mutation of linker PxSP sites confers resistance of Smad1 transcription activity to EGF inhibition.

At endogenous levels, mouse embryonic fibroblasts (MEFs) from wild-type and Smad1^{L/L} mice were used to study the effects of ERK activation on Smad1 activity [45]. FGF treatment of wild-type fibroblasts inhibited the BMP induced activities of the reporter gene BRE (BMP Response Element)-Luc, and the endogenous Id-1, SnoN, and alkaline phosphatase genes approximately by half. In Smad1^{L/L} MEFs, FGF treatment did not affect basal as well as BMP induced activities of BER-Luc, Id-1, SnoN, and alkaline phosphatase genes. This suggests that FGF inhibition of BMP target genes requires Smad1 linker phosphorylation by ERK. Interestingly, when Smurf1 was

knocked down in wild-type MEFs, FGF no longer inhibit the activities of BMP responsive genes, strongly supporting the notion that Smurf1 is an obligate mediator of ERK inhibition of Smad1 activity. Mechanistically, Smurf1 binding promotes Smad1 ubiquitination and excludes nucleoorin binding, leading to Smad1 degradation and/or nuclear exclusion.

Biological effects of Smad1 linker phosphorylation have also been studied at the organismal level. In the ectoderm of Xenopus embryo, BMP functions to expand epidermis and to inhibit neural induction. Therefore, inhibition of BMP signaling is essential for neural induction of the Xenopus embryos. Multiple signals, namely FGF, IGF and BMP antagonists, are required for inhibiting endogenous BMP signals. Among these, FGF and IGF inhibit BMP signaling by phosphorylating Smad1 in the linker region [46]. Notably, Smad1 EPSM is a much stronger inhibitor of neural differentiation than wild type Smad1, due to the fact that Smad1 EPSM is not phosphorylated by ERK and is resistant to the inhibition of endogenous or exogenous FGF and IGF signals. In addition, injection of wild type Smad1 mRNA into the animal pole of Xenopus embryos had a very mild ventralization effect. Endogenous ERK activity phosphorylates wildtype Smad1 to inhibit its activity. In contrast, Smad1 EPSM strongly ventralizes the *Xenopus* embryos. This is because Smad1 EPSM is resistant to ERK phosphorylation and inhibition. These findings in Xenopus embryos suggest that the functions of Smad1 in animal development are also inhibited by ERK phosphorylation.

To study the effects of Smad1 linker phosphorylation in mouse development, Smad1 linker mutant knock-in mice have been created [132]. In these mice (Smad1^{L/L}), the native Smad1 locus was replaced by a Smad1 mutated in the four ERK sites and one

GSK-3 site. Smad1^{L/L} mice survived emryogenesis, and were born with predicted mendelian ratios from heterozygous intercrosses. Despite relatively normal embryonic development, the homeostasis of gastric epithelium from Smad1^{L/L} mice were disrupted, characterized by a loss of the balance of numbers of different types of cells. Compared to wild type mice, the gastric epithelium of Smad1^{L/L} mice had less zymogenic cells and more parietal cells. How the Smad1 linker phosphorylation affects the differentiation of the gastric epithelium is unknown. In parietal cells from Smad1 L/L stomach, Smad1 protein is removed from the cytoplasm and accumulates in the nucleus and also on the plasma membrane. In wild-type parietal cells, Smad1 was distributed both in the cytoplasm and in the nucleus. Additionally, in gastric parietal cells from Smad1^{L/L} mice, the nuclear accumulation of β -catenin was enhanced. Whether enhanced nuclear β -catenin level accounts for the imbalance of zymogenic and parietal cell types in Smad1^{L/L} mutant mice remains to be investigated.

In addition to abnormal stomach, Smad1 linker mutant mice had less primordial germ cells (PGCs). It is known that TGF- β family members play important roles in specifying PGCs. It is not clear how the linker phosphorylation regulates the generation of PGCs.

Cell phenotypes of MEFs from Smad1^{L/L} mice are also abnormal compared with those from wild-type mice. Similar with that in parietal cells, linker mutant Smad1 also displayed the typical nuclear-plasma membrane localization pattern in MEFs. In addition to this, MEFs with mutant Smad1 had stronger cell-cell contacts than wild-type MEFs. In contrast to the adhesion zippers between cells observed in wild-type MEFs, Smad1^{L/L} MEFs are characterized by tight junctions between cells. Compared with wild-type

MEFs, actin cytoskeleton from Smad1^{L/L} MEFs showed more cortical actin but less stress fibers.

The analysis of the phenotypes of Smad1^{L/-} mice indicated that linker mutated Smad1 represents a hypomorphic allel rather than a hypermorphic allel. However, overexpression studies in cell culture and *Xenopus* embryos have demonstrated that Smad1 linker mutant has stronger transcription activities than wild-type Samd1, which predicts that Smad1^L should be a stronger allel than wild-type Smad1. In the knock-in mice, Smad1^L mutant protein was expressed at endogenous level. Linker region phosphorylation of endogenously expressed Smad1 may have additional functions besides inducing Smurf1 binding. These additional functions include regulation of cellular junction formation, regulation of actin cytoskeleton organization, regulation of β -catenin signaling, and the prevention of Smad1 retention at the plasma membrane. How the cellular effects of Smad1 linker phosphorylation is linked to its function in determining the cell fates of gastric epithelium and PGCs is not known.

Smad1 linker phosphorylation only modestly affected the association between Smad1 and Smad4, and these effects are more likely to be secondary to its effect on Smad1 cellular distribution. Whether Smad1 linker phosphorylation affects other aspects of Smad1 signaling remains an open question.

Smad2 and Smad3 have linker regions divergent in sequence from that of Smad1. None of the four PxSP motifs in the Smad1 linker region is conserved in Smad2 and Smad3 (Figure 5). Nonetheless, linker regions of Smad2/3 also contain Px(S/T)P motifs and xx(S/T)P motifs that lack the proline at the -2 position. Px(S/T)P motifs are consensus sites for ERK/MAPK, whereas xx(S/T)P motifs are consensus sites for proline-directed kinases, a broader category of kinases that also include ERK/MAPK. Smad2 contains one Px(S/T)P motif ($PET^{220}P$) and three xx(S/T)P motifs ($TGS^{245}P$, $ELS^{250}P$, and $TLS^{255}P$), whereas Smad3 contains two Px(S/T)P motifs ($PET^{179}P$, $PMS^{213}P$) and two xx(S/T)P motifs ($AGS^{204}P$, $NLS^{208}P$).

In the mouse mammary epithelial cell line EpH4, TGF- β treatment induces growth inhibition. In an EpH4 derived cell line stably overexpressing the v-Ha-Ras (the EpRas cell line), TGF- β treatment induces a fibroblastoid phenotype but does not lead to growth inhibition [48]. The TGF- β induced activation of the reporter gene (ARE)₃-Luc was significantly diminished in EpRas cells than in EpH4 cells. The TGF- β induced C-tail phosphorylation of Smad2 was similar in EpH4 cells and in EpRas cells. To probe the mechanism of abolishment of TGF- β growth inhibitory effect by Ras signaling in EpRas cells, endogenous ³²P labeled Smad2/3 was significantly higher in EpRas cells than in EpH4 cells. This suggests that hyperactive Ras signaling may inhibit Smad2/3 activity by inducing Smad2/3 phosphorylation.

When transfected into Mv1Lu/R1B-L17 cells (a Mv1Lu derived cell line that is deficient in T β RI), Smad2 and Smad3 can be significantly phosphorylated upon EGF treatment, or upon co-expression of RasV12 (a constitutively active form of Ras) or constitutively active (ca)-MEK1. Pre-treatment of cells with PD98059, a MEK inhibitor, strongly inhibited Smad2/3 phosphorylation. Furthermore, recombinant Smad2/3 can be directly phosphorylated by recombinant ERK2 *in vitro*. To map phosphorylation sites in Smad2/3 that are induced by the Ras pathway, endogenous Smad2/3 labeled by ³²P was immunoprecipitated and subjected to tryptic digestion. The size of ³²P labeled tryptic

peptide was more than 13 KD (kilo-dalton) on SDS-PAGE and matches with the predicted size of the linker region in the tryptic digestion. Thus, potential phosphorylation sites reside in the linker regions of Smad2/3. When the serine/threonines (S/T) in the four (P-x-)(S/T)-P motifs in Smad2/3 linker regions were mutated to alanine or valine, phosphorylation of Smad2/3 *in vivo* in the presence of RasV12, caMEK1, or EGF, or *in vitro* by recombinant ERK2, was almost abolished. In contrast, when the C-tail SSxS motif was mutated, Smad2/3 were still phosphorylated upon EGF stimulation or RasV12 overexpression. These findings suggest that ERK downstream of Ras pathway phosphorylates Smad2/3 mainly on four (P-x-)(S/T)-P motifs in the linker region independent of the C-terminal motif.

As revealed by an antibody recognizing both Smad2 and Smad3, Smad2/3 were evenly distributed in the cytoplasm and nucleus in the absence of TGF- β in EpH4 cells. Upon TGF- β treatment, significant nuclear accumulation of Smad2/3 was observed. Remarkably, in the v-Ha-Ras overexpressing EpRas cells, TGF- β induced Smad2/3 nuclear localization was greatly impaired. In Mv1Lu cells, a highly TGF- β responsive mink lung epithelial cell line, TGF- β treatment significantly induces the nuclear accumulation of Smad2/3. Notably, transfection of the constitutively active Ras, RasV12, or constitutively active MEK1 (caMEK1) into Mv1Lu cells strongly inhibited the TGF- β induced nuclear accumulation of endogenous Smad2/3 in transfected cells. Consistent with studies involving overexpressed Ras or MEK, pretreatment of Mv1Lu cells with EGF partially blocked TGF- β induced Smad2/3 nuclear translocation. In addition, in a series of colon cacinoma cell lines, the presence of constitutively active Ras allel correlated with poor nuclear accumulation of Smad2/3 upon TGF- β stimulation. Taken together, these findings suggest that active Ras/MEK/ERK pathway inhibits Smad2/3 nuclear accumulation in response to TGF-β tretment.

To investigate whether ERK inhibition of Smad2/3 nuclear localization was due to phosphorylation in the linker region of Smad2/3, linker phosphorylation site mutant Smad3 (Smad3 EPSM) was examined for their TGF- β induced nuclear translocation. In the presence of caMEK1, nuclear localization of wild-type Smad3 in response to TGF- β was completely blocked. In contrast, Smad3 EPSM readily accumulated in the nucleus upon TGF- β treatment even in the presence of caMEK1. Thus, active ERK signaling blocks Smad2/3 nuclear accumulation through phosphorylation of Smad2/3 linker region.

When transfected into EpRas cells, Smad3 EPSM restored the response of $(ARE)_{3}$ -Luc to TGF- β , whereas wild-type Smad3 only modestly increased the activity of $(ARE)_{3}$ -Luc. Smad3 EPSM also restored the growth inhibition induced by TGF- β in EpRas cells. The wild-type Smad3 only slightly inhibited cell growth in response to TGF- β . Thus, the overactive Ras pathway in EpRas ceclls overrides the anti-proliferative activity of endogenous Smad2/3 and that of transfected wild-type Smad3, but does not affect the anti-proliferative activity of a phosphorylation-resistant mutant Smad3, Smad3 EPSM. In summary, ERK induced Smad2/3 linker phosphorylation inhibits the antiproliferative activities of Smad2/3 through Smad2/3 nuclear exclusion.

In *Xenopus* development, the gastrula ectoderm can respond to activin signals by expressing mesodermal genes up to stage 10.5. Then after stage 11, ectoderm cells suddenly lose this ability to express mesodermal genes upon activin treatment. The sudden loss of competence is characterized by a global loss of activin induced

expression of target genes such as Xnr1, Chordin, Eomes, goosecoid, and X brachyury, suggesting that activin signaling *per se* is completely blocked. Green fluorescence protein (GFP) tagged Samd2 was expressed in *Xenopus* embryos. At stage 10.5, GFP-Smad2 translocated into the nucleus of animal pole cells in response to activin treatment. After stage 11, however, activin treatment cannot induce the nuclear accumulation of GFP-Smad2 in animal caps. Thus, the competence of Xenopus ectoderm cells to respond to activin treatment is lost after embryonic stage 11 due to the inability of Smad2 to translocate into the nucleus in response to activin [133].

What is the mechanistic basis for the Smad2 nuclear exclusion after stage 11? ³²P labeling of myc-tagged Smad2 (wild-type) overexpressed in Xenopus embryos revealed that there was a sudden increase of phosphorylation on Smad2 at stage 11. The coincidence of the timings of Smad2 phosphorylation increase and the onset of Smad2 nuclear exclusion suggests that there may be a causal link between Smad2 phosphorylation and nuclear exclusion. Smad2 contains several xx(S/T)P motifs in the linker region that are targets for proline-directed protein kinases. To test the hypothesis that Smad2 phosphorylation in the linker region causes its nuclear exclusion, mutant Smad2 was made in which three (S/T)-P motifs (S²⁴⁵P, S²⁵⁰P, S²⁵⁵P) in Smad2 were replaced by alanines. Mutant Smad2 (designated Smad2 3AP) was not phosphorylated at or after stage 11, and strikingly, GFP-Smad2 3AP continues to translocate to the nuclei of *Xenopus* embryo cells in response to activin even after stage 11, the time point when the wild-type Smad2 was kept out of the nucleus through linker phosphorylation. Thus, Smad2 linker phosphorylation is required for the blokade of nuclear accumulation. In conclusion, both in certain mammalian tissue culture cells and in the developing *Xenopus* embryos, Smad2 phosphorylation in the linker region blocks its nuclear accumulation upon TGF- β /activin stimulation.

Evidences showed that an endogenous signal induces the phosphorylation of Smad2 on three linker xx(S/T)P motifs (S²⁴⁵P, S²⁵⁰P, S²⁵⁵P) and leads to the inhibition of activin induced Smad2 nuclear accumulation in *Xenopus* embryos after stage 11 of embryonic development. The nature of this endogenous signal is unkown. Also unknown is the kinase(s) that phosphorylate(s) the three ((S/T))-P sites in Smad2. Since (S/T)-P motifs are consensus sites for proline-directed kinases that include ERK/MAPK, the endogenous kinase might be ERK or a related kinase.

The inhibition of nuclear translocation of Smad2/3 by linker region phosphorylation parallels the inhibition of Smad1 nuclear accumulation through linker phosphorylation. Thus, the four (P-x-)(S/T)-P motifs in the Smad2/3 linker region are functionally equivalent to the four PxSP motifs in the Smad1 linker region, at least in the *Xenopus* embryos and certain tissue culture cell lines. Smad1 nuclear exclusion induced by linker phosphorylation is mediated by Smurf1. What is the mechanism of inhibition of Smad2/3 nuclear accumulation by linker phosphorylation? Does it also require the endogenous Smurf1 or the related Smurf2? These questions remain to be tested by further experiments.

The inhibition of Smad2/3 nuclear accumulation by linker phosphorylation is highly context dependent. In different cell types, the extent of the inhibition of Smad nuclear translocation by linker phosphorylation varies greatly. Smad2 nuclear exclusion by linker phosphorylation is most prominent in *Xenopus* embryos. In the mouse mammary epithelial cell line EpH4, Smad2/3 accumulates in the nucleus upon TGF-β treatment.

But in the EpH4 derived cell line EpRas that overexpress v-Ras, the TGF- β induced Smad2/3 nuclear localization was almost completely blocked. A similar correlation was found in a series of colon cacinoma cell lines: cell lines with constitutively active Ras allel have poor Smad2/3 nuclear accumulation in response to TGF- β , while in cell lines with wild-type Ras allel the nuclear accumulation of Smad2/3 was normal. In Mv1Lu, overexpression of RasV12 or caMEK1 abolish Smad2/3 nuclear accumulation whereas pre-treatment with EGF, a relatively weaker ERK activator, partially blocks TGF-β induced Smad2/3 nuclear accumulation. However, in HaCaT cells, stable overexpression of a constitutively active MEK (MEK O56P) did not affect TGF-β induced Smad3 nuclear accumulation [134]. Another study using madin-darby canine kidney cells (MDCK cells, a dog kidney epithelial cell line) showed that inducible expression of constitutively active Raf or stable expression of RasV12 did not inhibit or increase the TGF- β induced nuclear translocation of Smad2, Smad3, and Smad4 [135]. Interestingly, when a constitutively active N-Ras (N-Ras^{61K}) was stably expressed at different levels in Mv1Lu cells, different effects on Smad2/3 nuclear localization were observed [136]. Whereas lower expression level (~2 fold the endogenous level) of N-Ras^{61K} did not block Smad2/3 nuclear translocation, a higher expression level (~5 fold endogenous level) significantly inhibited TGF- β induced Smad2/3 nuclear accumulation. Thus, the effects of ERK signaling on Smad2/3 subcellular localization depend on both the cell type and the dose of Ras/MEK/ERK pathway activation.

Surprisingly, treatment of HepG2 cells by HGF, an activator of Ras/ERK pathway, induced the nuclear translocation of Smad2 in the absence of TGF- β [49]. Two-dimensional electrophoresis of ³²P labeled tryptic peptide revealed that HGF induces

phosphorylation of Smad2 on residues distinct from the SSxS motif. Nonetheless, phosphorylation of Smad2 by HGF treatment requires an intact C-tail. HGF induced Smad2 phosphorylation was readily inhibited by pre-treatment of cells with a MEK inhibitor, PD98059. The HGF induced phosphorylation sites in Smad2 was not mapped, and it remains to be determined whether HGF induced Smad2 phosphorylation occurs in the four (P-x-)(S/T)-P sites in the linker region. In MDA-MB-468 cells transfected with exogenous Smad4 expression vector, or in the Mv1Lu derived L17-R1B cells, HGF treatment of cells reproducibly caused activation of 3TP-Luc reporter gene (a TGF- β responsive reporter gene construct) in the absence of autocrine TGF- β signaling was blocked by dominant-negative T β RII or TGF- β neutralizing antibody. These results were consistent with the observation that nuclear accumulation of Smad2 can be induced by HGF in the absence of TGF- β signaling in HepG2 cells.

It is necessary to point out that activation of the ERK pathway by HGF/EGF induces the expression of c-fos, a component of the AP-1 transcription factor. Thus, Ras/MEK/ERK pathway activation could activate AP-1 transcriptional activity. It has been reported that AP-1 transcription factor physically interact with Smad3 and cooperate with Smad3 and Smad4 to activate a subset of TGF- β target genes such as collagease I gene, and PAI-1 [137]. The 3TP-Luc reporter gene contains the AP-1 binding sites from both collagenase I promoter and the PAI-1 promoter. AP-1 can enhance transcription activity of Smad3/Smad4. Thus, although it is possible that the activation of 3TP-Luc by HGF treatment is through Smad2 phosphorylation and nuclear accumulation, it is also possible that HGF induced increase of 3TP-Luc activity is nonspecific effect due to the co-activation of endogenous Smad3 and Smad4 by the AP-1 transcription factor. Future experiments should clarify this issue.

The kinase that phosphorylates Smad2 is located downstream of MEK, an activator of ERK. Whether the Smad2 kinase is ERK or is located further downstream is unknkown yet. Also unknown are the exact phosphorylation sites induced by HGF/EGF. Nonetheless, the Smad2 3SA mutant (in which the C-terminal SSMS motif was changed to AAMA) lost the HGF/EGF induced phosphorylation outside the SSMS motif, despite the fact that the phosphorylation sites by HGF/EGF were not directly mutated. Thus, Smad2 3SA is a potential dominant-negative inhibitor of HGF/Smad2 signaling. When Smad2 3SA was co-transfected with the TGF- β responsive reporter 3TP-Luc into L17-R1B cells, the HGF induced activation of 3TP-Luc was significantly blocked. Consistent with the functional assay, Smad2 3SA did not translocate into nucleus when cells were treated with HGF, although the wild-type Smad2 did accumulate in the nucleus under the same condition.

HGF promotes Smad2 phosphorylation only when the C-terminal SSMS motif is intact, although C-tail itself is not phosphorylated. This requirement is unique for HGF induced Smad2 phosphorylation in HepG2 cells. HGF induces phosphorylation of Smad1 in HepG2 cells even when the C-tail SSVS motif was mutated to AAVA [47]. Smad1 phosphorylation in the linker region by ERK either in *Xenopus* embryos or in R1B-L17 cells does not require the C-terminal SSVS motif. Smad2/3 phosphorylation in the linker region by ERK in R1B-L17 also can occur in the absence of the C-terminal SSxS motif. In conclusion, phosphorylation of Smad1/2/3 linker region by ERK does not require the C-terminal motif in most cases, but HGF induced Smad2 phosphorylation requires an intact C-tail motif.

Notably, the nuclear accumulation of Smad2 in HepG2 cells in response to TGF- β was partially impaired when the cells were pre-treated with the MEK inhibitor PD98059. This suggested that endogenous MEK/ERK activity is required for TGF- β induced Smad2 nuclear translocation in HepG2 cells. The underlying mechanism of this requirement is unknown. To establish a link between Smad2 phosphorylation and nuclear translocation, the phosphorylation sites in Smad2 should be mapped, and the ability of phosphorylation mutant Smad2 to translocate to the nucleus in response to HGF or MEK-A should be examined.

It was also reported that overexpression of constitutively active MEK (MEK-A) in HepG2 cells partially induced Smad2 nuclear accumulation in the absence of TGF- β [138]. Activation of ERK pathway could promote TGF- β production and induce autocrine TGF- β signaling. Thus, MEK-A induced Smad2 nuclear accumulation could be an indirect effect through autocrine TGF- β signals. Alternatively, it is also possible that MEK-A expression activates ERK, which phosphorylates Smad2 and induces its nuclear translocation.

In summary, activation of the ERK/MAPK pathway either inhibits, or has no effect, or promotes Smad2/3 nuclear accumulation in various cell types such as *Xenopus* gastrula ectodermal cells, EpH4 and EpRas (mouse mammary epithelial cells), Mv1Lu (mink lung epithelial cell line), MDCK (dog kidney epithelial cell line), HaCaT (human epithelial keratinocytes), or HepG2 (heptoma cells). A causal link between Smad2/3 linker phosphorylation by ERK and inhibition of Smad2/3 nuclear accumulation in

response to TGF- β has been established by examining the behavior of Smad2/3 phosphorylation resistant mutants (Smad2/3 EPSM, or Smad2 3AP). However, the mechanisms of the promotion of Smad2 nuclear accumulation by HGF treatment or MEK-A overexpression have not been fully investigated. Specifically, whether HGF or MEK-A induced Smad2 nuclear translocation requires phosphorylation on potential (S/T)-P motifs is not known.

In addition to nuclear accumulation, Smad phosphorylation by ERK can also influence other aspects of TGF-β signaling. In COS-7 cells, EGF treatment induced a significant increase in the phosphorylation level of overexpressed Smad2 [139]. This observation is consistent with previous findings that ERK activated by growth factors can phosphorylate R-Smad on (P-x-)(S/T)-P motifs in their linker regions. Interestingly, tryptic digestion of Smad2 *in vitro* phosphorylated by ERK1 followed by separation of peptides using HPLC (high performance liquid chromatography) revealed two peaks of radioactivity. N-end sequencing revealed that one peak of radioactivity corresponded to the Smad2 linker region, whereas the other peak corresponded to the N-terminal peptide. The only (S/T)-P motifs and T8 in the MH1 domain of Smad2 nearly abolished EGF induced phosphorylation. This study expanded ERK phosphorylation sites in Smad2 to include not only the linker region, but also one site in MH1 domain.

In Mv1Lu/R1B-L17 cells, the activity of $(ARE)_3$ -Luc can be dramatically increased by co-transfection of the constitutively active T β RI, ALK5 (TD), or Smad2 (2E), a constitutively active Smad2 that has the C-terminal SSMS changed to SEME to mimick phosphorylation. Interestingly, co-transfection of constitutively active MEK1, MEK1 (ED), futher increased (ARE)₃-Luc activity activated by ALK5 (TD) or Smad2 (2E). Previously it was found that ERK pathway represses R-Smad activity by inhibiting their nuclear translocation. However, in this cell type and context, MEK1 (ED) promoted TGF-B/Smad2 signaling. Examination of Smad2 protein half-life in the absence or presence of constitutively active MEK1 (MEK1*) revealed that expression of MEK1* significantly slowed down the degradation of Smad2 and increased steady state Smad2 level. On the contrary, co-expression of the ERK phosphatase HVH2 decreased Smad2 level. This suggested that ERK mediated phosphorylation increases Smad2 protein stability. Following up this phenomenon, mutants of Smad2 were made that abolish or mimick phosphorylation on the ERK targeted sites. In COS-7 cells, alanine and/or valine substituted mutants of Smad2 have much lower steady state protein level than mutants with aspartate (phosphorylation mimicking) mutations. As a result, Smad2 mutants with ERK sites substituted by alanine/valine have much lower activities on (ARE)₃-Luc than wild-type Smad2 due to their low protein stability. On the other hand, Smad2 mutants with aspartate substitution showed much stronger activities on (ARE)₃-Luc reporter than wild-type Smad2, due to increased stability. Smad2 phosphorylation by ERK did not directly affect their heteromerization with Smad4, since wild-type or phosphorylation mimicking mutants of recombinant GST-Smad2 bind Smad4 with similar affinity. However, when wild-type or phosphorylation mimicking mutants of Smad2 were coexpressed with Smad4 in cells, phosphorylation mimicking Smad2 mutants pulled down more Smad4 simply because of their higher protein level.

Smad2 physically interacts with calmodulin, a protein that binds Ca^{2+} and mediates many functions of Ca^{2+} signaling. The ERK site in MH1 domain of Smad2 (PFT⁸P)

overlaps with calmodulin binding domain of Smad2. Calmodulin inhibits Smad2 phosphorylation by ERK in *in vitro* kinase assay. Whether calmodulin only inhibits phosphorylation on T8 site or it inhibits Smad2 phosphorylation on all (S/T)-P sites is unknown. When overexpressed in cells, calmodulin decreased the protein level of co-expressed Smad2 in a dose-dependent manner, consistent with the finding that decreased phosphorylation of Smad2 led to decreased stability. Thus, the newly identified ERK site T8 in Smad2 mediated at least some of the effects of calmodulin on Smad2 signaling.

Notably, in other studies, the steady-state level of Smad2 and Smad3 were not found to change in response to the expression of v-Ha-Ras or RasV12 in the mouse mammary epithelial cell system EpH4/EpRas or the Mv1Lu/L17-R1B cells. The steady-state levels of wild-type Smad2 or 3 were very similar to those of Smad2 and Smad3 EPSM. Similarly, the nuclear level of Smad2 and Smad3 did not show significant difference in MDCK cell line or its Ras or Raf overexpressing derivatives. Thus, the effects of ERK phosphorylation on Smad2 and Smad3 protein stability are highly cell line and context dependent. In addition, the high level of overexpression of Smad2/3 and their mutants may mask their differences in stability.

In human glomerular mesangial cells, TGF- β treatment activated both the Smad pathway and the ERK1/2 pathway. TGF- β activated T β RI and ERK kinases phosphorylated Smad3 on the C-terminal SSVS motif and the linker region, respectively [140]. In this context, The ERK sites in Smad3 were within the four (P-x-)(S/T)-P motifs in the linker region. Phosphorylation of Smad3 linker region was inhibited by pre-treatment with MEK inhibitors PD98059 or U0126. Remarkably, MEK inhibitor pre-treatment also partially blocked the TGF- β induced Smad3-Smad4 heteromerization,

suggesting that ERK mediated Smad3 linker phosphorylation is required for Smad3/4 association in human glomerular mesangial cells. Interestingly, inhibition of ERK activity did not affect TGF- β induced Smad3/4 association in the mammary epithelial cell line NMuMG and the human keratinocytes HaCaT cells, suggesting that the requirement of ERK activity for Smad3/4 heteromerization is cell type dependent. Overexpression of a constitutively active (ca-) MEK enhanced TGF- β induced α 2(I) collagen promoter activity. The activity of Smad3 3SA mutant (in which the SSVS C-tail was mutated to AAVA) towards α 2(I) collagen promoter was significantly increased in the presence of ca-MEK, whereas activity of Smad3 EPSM (in which the four linker (P-x-)(S/T)-P motifs were mutated to alanine/valine) on the α 2(I) promoter was not affected by ca-MEK expression. The results suggest that ERK phosphorylation of Smad3 linker regiopn, but not the C-tail, enhances its transcription activity on the α 2(I) collagen promoter.

In conclusion, regulation of Smad2/3 by the Ras/MEK/ERK pathway is highly context dependent. Distinct steps in TGF- β /Smad signaling, such as Smad2/3 nuclear accumulation, Smad3-Smad4 heteromerization, or protein stability, may be affected in different cell types.

Notably, TGF-β itself activates ERK/MAPK in certain cell lines. For example, TGFβ induces phosphorylation and activation of ERK in human glomerular mesangial cells but not in mammary epithelial cell line NMuMG [140]. TGF-β induced ERK activity in mesangial cells was required for Smad3-Smad4 complex formation in mesangial cells. Whether ERK induced Smad3-Smad4 heteromerization through linker phosphorylation of Smad3 remains to be determined. In HepG2 cells, TGF-β induces prolonged ERK activation that peaks at 2 hours after adding TGF- β . Inhibition of ERK activation impairs TGF- β induced Smad2 nuclear accumulation. Whether TGF- β activated ERK promoted Smad2 nuclear localization through linker phosphorylation remains to be further investigated. In HaCaT cells, TGF- β treatment of cells dramatically increased the activity of GAL4-ELK1, a fusion protein that reflects cellular ERK activity, on a reporter containing five copies of GAL4 binding sequence [134]. Inhibition of endogenous ERK activity abolished the TGF- β induced expression of the CDK inhibitors p21WAF1/Cip1 and p15INK4B, although the TGF- β induced Smad3 nuclear localization, Smad3-Smad3 complex formation and p300-Smad3 interaction were not affected. The findings that TGF- β activates ERK pathway which in turn modulates Smad2/3 activity through linker phosphorylation-dependent or independent mechanisms suggest that ERK may be an integral part of TGF- β signaling in certain cellular contexts.

In several studies, the overexpression of ERK pathway components was used to study the effects of ERK-mediated Smad2/3 linker phosphorylation on their activities. Caution must be used to interpret the results of these experiments. Overexpression of Ras/MEK/ERK pathway components may induce the production of TGF- β and promote autocrine TGF- β signaling. Hyper-activation of the Ras/MEK/ERK pathway can also strongly activate the AP-1 transcription factor, which enhances Smad signaling. Thus, overexpression of Ras, MEK or ERK could non-specifically activate TGF- β /Smad signaling. One way to avoid being misled by the non-specific effects of protein overexpression is to use mutants in which the phospho-rylation sites have been mutated to alanine/valine. To make phosphorylation-resistant mutants, the phosphorylation sites must be precisely mapped.

In HaCaT (human keratinocytes) and Mv1Lu (mink lung epithelial cells) cells, which are highly responsive to TGF-β, the EGF induced phosphorylation sites on Smad3 was mapped to T179, S204, and S208 [154]. Among these three sites, S208 was by far the most sensitive site. This is consistent with the prediction of the computer program Scansite. Under high stringency, S208 is the only site in the Smad3 linker region predicted to be phosphorylated by ERK/MAPK. Mutation of these three sites to non-phosphorylatable residues significantly increased the activity of Smad3 on the reporter (ARE)₃-Luc. The data is consistent with an inhibitory role of EGF induced Smad3 linker phosphorylation on its transcription activity.

Regulation of Smad signaling by ERK pathway also occurs through mechanisms independent of Smad2/3 linker phosphorylation. The AP-1 transcription factor comprises the Jun family and the fos family of transcriptional factors. Activation of Ras/MEK/ERK pathway induces the expression of c-fos. Thus, active Ras/MEK/ERK pathway increases the cellular activity of AP-1 transcription factor. The AP-1 transcription factors, c-Jun and c-fos, physically interact with Smad3 [137]. In cells, a complex of Smad3/4-c-jun-c-fos is assembled on the AP-1 binding site of the collagenase I promoter in response to TGF- β treatment. The collagenase I AP-1 binding sites contain overlapping c-jun and Smad3/4 binding elements. Both Smad3/4 and c-Jun/c-fos in this complex can recruit co-activators to promote transcription from the collagenase I promoter. Moreover, Smad3/4 can trans-activate transcription from AP-1 sites in the absence of Smad binding elements. c-Jun and c-fos also trans-activate GAL4-Smad3 activity from a promoter containing five copies of GAL4 binding sites. Thus, the physical interaction between Smads and AP-1 factors enhance each other's transcriptional activities. These findings from literature reminded people that observations in previous studies may have included some non-spcific effects. In previous work, to study the effect of R-Smad linker phosphorylation on their activity, the authors activated ERK pathway by treating cells with HGF/EGF or by overexpressing constitutively active components of the Ras/MEK/ERK pathway. The observed effects were the combination of the indirect effects on Smad activity through the activation of AP-1 transcription factors and the direct effects of R-Smad linker phosphorylation on Smad activity. In this view, loss of function assays such as using specific MAPK inhibitors, expressing dominant-negative signaling molecules, or creating mutants in which the phosphorylation sites are mutated may yield more reliable results.

In one study, overexpression of the constitutively active MEK1, MEK1 (S218/S222D), in HaCaT cells dramatically increased both the basal and TGF- β induced activities of p21WAF1/Cip1 and p15INK4B promoter reporters. Nonetheless, the pre-treatment of cells with the MEK inhibitor U0126 did not affect TGF- β induced Smad3-Smad4 complex formation, and the TGF- β induced Smad3-p300 interaction. The stable overexpression of MEK1 Q56P (a constitutively active MEK1) did not affect the TGF- β induced Smad3 nuclear accumulation and DNA binding activity of Smad3. Blocking endogenous ERK activity using U0126 abolished the induction of p15 and p21 by TGF- β . In this study, MEK/ERK activity is required for TGF- β induced p15 and p21 expression through a linker phosphorylation-independent mechanism.

1.5.3 c-Jun N-terminal Kinase (JNK)

c-Jun N-terminal Kinases (JNKs) belong to the MAPK superfamily. JNK is activated by growth factors (e.g. HGF), cytokines (e.g. TNF- α), and stress signals (e.g. UV, osmotic imbalance). JNK phosphorylates downstream transcription factors such as c-Jun and ATF-2, leading to their activation. JNK is an inducer of apoptosis, but may also play a role in cell survival.

Interestingly, JNK is also activated by TGF- β [145]. In different cellular contexts, TGF- β treatment can lead to delayed, sustained JNK activity or quick, transient JNK activation. In MDCK (Madin-Darby Canine Kidney) cells, TGF- β induced sustained JNK activation that peaked about 8 hours after TGF- β addition. On the contrary, TGF- β induced JNK activity was maximum at 15 minutes in RGM-1 cells (normal rat gastric epithelial cell) and HaCaT cells. The transient activity of JNK goes back to basal level by one hour. Interestingly, TGF- β treatment of Mv1Lu cells led to biphasic activation of JNK, a primary peak at 15 minutes and a secondary peak after 8 hours. The primary JNK activity was independent of Smad whereas the delayed peak required the presence of Smad4.

The mechanisms of JNK activation in response to TGF- β are poorly understood. A number of reports implicated MEKK-1 (MAPK/ERK-Kinase Kinase-1), TAK1 (TGF- β Activated Kinase-1), or Rho family small GTPases in the activation of JNK by TGF- β treatment. The details of TGF- β induced JNK activation are largely unknown.

In the linker region of Smad2, there are one P-x-(S/T)-P site (PET²²⁰P) and three x-x-(S/T)-P sites (TGS²⁴⁵P, ELS²⁵⁰P, TLS²⁵⁵P). In the Smad3 linker region, there are two Px-(S/T)-P sites (PET¹⁷⁹P, PMS²¹³P) and two x-x-(S/T)-P sites (AGS²⁰⁴P, NLS²⁰⁸P). The P-x-(S/T)-P and x-x-(S/T)-P motifs are phosphorylation sites for proline-directed protein kinases, which include the MAPK superfamily. Thus, these sites are potential targets for JNK, which belongs to MAPK superfamily. Smad1, Smad2, and Smad3 were all found to be phosphorylated by JNK *in vivo* and/or *in vitro*.

In human keratinocytes HaCaT cells, UV irradiation and hypertonic NaCl solution treatment induced the activation of both JNK and p38 MAPK. Notably, the linker region of Smad1 was also phosphorylated after the stresses (UV and hypertonic NaCl treatments), revealed by the S206 site-specific phospho-peptide antibody and an antibody recognizing phosphorylated PxS*P motif ("*" designates the phosphorylated serine). The UV induced Smad1 linker phosphorylation was inhibited by combined treatment by the JNK inhibitor SP600125 and the p38 inhibitor SB203580, suggesting that JNK and p38 jointly phosphorylated Smad1 linker region in response to UV stress [45].

In Mv1Lu cells, TGF- β activated JNK through the small GTPases RhoA and Rac1 [145]. Notably, endogenous JNK immunoprecipitated from lysates of Mv1Lu cells following TGF- β treatment can phosphorylate recombinant Smad3 *in vitro*. In this report, the phosphorylation sites were not mapped. Phosphorylation of Smad2/3 was examined in a series of studies [142-144] using phospho-specific antibodies raised against phosphorylated Smad2 S250/S255 and phosphorylated Smad3 S208/S213. JNK activated by HGF and/or TGF- β immunoprecipitated from RGM-1 cells (normal rat gastric epithelial cells) can phosphorylate recombinant Smad2 and Smad3 *in vitro* on S250/S255 and S208S213 respectively. Similarly, in hepatic stellate cells (HSCs) treated with PDGF (platelet-derived gowth factor) and/or TGF- β , JNK immunoprecipitated from the cell lysates was able to phosphorylate both Smad2 and Smad3 *in vitro* on

S250/S255 and S208/S213. In addition, JNK immunoprecipitated from colon cancer tissues was shown to directly phosphorylate Smad2/3 on S250/S255 and S208/S213. Whether JNK phosphorylates other sites in the Smad2/3 linker region besides S250/S255 and S208/S213 was not examined, due to lack of appropriate phospho-specific antibodies.

In Mv1Lu cells, TGF- β treatment induces the labeling of transfected Smad3 by exogenously applied ³²P. Co-expression of wild-type JNK with Smad3 strongly increased incoporation of ³²P into Smad3, both in the basal state and in the TGF- β stimulated state. On the contrary, expression of a dominant-negative JNK (JNK^{APF}) decreased the level of Smad3 phosphorylation both in the absence and in the presence of TGF- β .

In RGM-1 cells, endogenous Smad2 and Smad3 can be phosphorylated in S250/S255 and S208/S213 following treatment of cells by HGF and/or TGF- β . HGF and TGF- β cooperate to activate JNK. In a model of acute liver injury by CCl4 adminstration, PDGF and TGF- β activated JNK in hepatic stellate cells (HSCs), whose activity correlated with the phosphorylation of endogenous Smad2/3 on S250/S255 and S208/S213 respectively. In the development of colorectal carcinoma, JNK activity gradually increased along the different stages of colorectal cancer, namely normal colorectal epithelial cells, adenoma, adenocarcinoma, and invasive carcinoma. The phosphorylation of Smad2 and Smad3 linker region also significantly increased along the adenoma-carcinoma sequence. Overall, in acute liver injury model as well as in colorectal cancer development model, variation of JNK activity correlated with the change of phosphorylation of the Smad2/3 linker region, suggesting that JNK may be the

kinase that phosphorylates Smad2/3 linker region in these cases. This hypothesis need to be examined by experiments.

Phosphorylation of Smad2 and Smad3 in the linker region in RGM-1 cells following HGF or TGF- β treatment was strongly inhibited by the JNK inhibitor SP600125. Thus, it is likely that JNK phosphorylates the Smad2/3 linker region following treatment of cells by HGF and/or TGF- β . It is worth pointing out that SP600125 can inhibit 13 out of 28 kinases tested with equivalent or even better potency than it towards JNK. Therefore, caution should be used in attributing phosphorylation of a substrate to JNK simply because SP600125 can inhibit the phosphorylation.

JNK phosphorylation of Smad3 was also examined by using purified recombinant JNK. To evaluate whether Smad3 is a good substrate for JNK, the kinetic parameters of the kinase reaction containing JNK and Smad3 were calculated and compared to those obtained in kinase reaction containing JNK and its canonical substrate GST-ATF2 (1-109). The Vmax/Km value is a good indicator of how well a substrate can be phosphorylated by a kinase. The Vmax/Km value of GST-ATF2 (1-109) is ~40 fold more than that of Smad3 (our unpublished data). Thus, Smad3 is only a modestly-good substrate of JNK.

In many cell lines, such as RGM-1 (normal rat gastric epithelial cells) and Mv1Lu cells, the primary acivation of JNK by TGF- β is very transient, peaking at 15 minutes after TGF- β addition and declining to basal level after 1 hour. However, the Smad3 linker phosphorylation sustained much longer than 1 hour after TGF- β administration. Notably, p38 showed slow but prolonged activation by TGF- β that peaks at 1 hour in several cell lines such as RGM-1, Mv1Lu, and HaCaT. It is postulated that JNK serves

to initiate Smad3 linker phosphorylation after TGF- β stimulation, whereas p38 phosphorylates Smad3 at later time points, when JNK activity is low. This hypothesis has not been tested yet. Compared with Smad3, the linker phosphorylation of Smad2 was transient and was already declining by 1 hour. This is consistent with the fact that Smad2 cannot be phosphorylated by p38 (see below, 1.5.4 p38 MAPK), whose activity only reaches maximum after 1 hour of TGF- β treatment.

Phosphorylation of Smad2/3 linker region induced by JNK is independent of the Cterminal SSxS motif. When Smad3 3SA (C-terminal SSVS mutated to AAVA) were transfected into Mv1Lu cells and the cells was labeled by ³²P, TGF- β induced Smad3 3SA phosphorylation as well as it did wild-type Smad3. Overexpression of JNK further increased the phosphorylation on Smad3 3SA. Thus, JNK may phosphorylate Smad3 outside of the SSxS motif, and JNK induced phosphorylation of Smad3 can occur independent of C-tail. In a separate study, the overexpression of MEKK-1, an upstream activator of JNK, led to Smad2 phosphorylation even when the C-terminus of Smad2 was deleted.

Immunostaining with pSmad2 (S250/S255) and pSmad3 (S208/S213) antibodies (the antibodies target phosphorylated Smad2 S250/S255 and phosphorylated Smad3 S208/S213 sites, respectively) revealed that linker phosphorylated Smad2 localized to the cytoplasm whereas linker phosphorylated Smad3 exclusively accumulated in the nucleus in RGM-1 cells, activated HSCs, and colorectal cancer tissues. At lower concentration of linker phosphorylation, Smad2 linker phosphorylation may lead to Smad2 cytoplasmic retention whereas Smad3 linker phosphorylation promotes its nuclear accumulation. Consistent with this notion, induction of Smad3 linker

phosphorylation by HGF treatment strongly decreased TGF- β induced Smad3 Cterminal phosphorylation in RGM-1 cells. This is presumably a result of sequestration of Smad3 into the nuclei and limited access to the membrane-bound type I receptor. On the contrary, co-treatment of RGM-1 cells with TGF- β and HGF induced stronger C-tail phosphorylation of Smad2 than TGF- β treatment alone. This is likely because HGF induced linker phosphorylation led to cytoplasmic retention of Smad2 and better access of Smad2 to type I receptor.

The effects of linker phosphorylation on Smad2/3 subcellular localization were also studied by overexpression of JNK or its upstream activator MEKK-1. When overexpressed in Mv1Lu cells, Smad3 3SA did not translocate to the nucleus in response to TGF- β treatment, due to the absence of phosphorylation sites for T β RI. Remarkably, when JNK was co-expressed with Smad3 3SA, TGF- β treatment induced the nuclear accumulation of Smad3 3SA [145]. In addition, MEKK-1 co-expression with Smad2 induced the nuclear accumulation of Smad2 in the absence of TGF- β . Thus, when the linker region was heavily phosphorylated by overexpressed JNK, both Smad2 and Smad3 translocated into the nucleus in the absence of C-terminus phoshorylation. In conclusion, linker phosphorylation of Smad3 by JNK promotes its nuclear accumulation, whereas for Smad2, lower level of linker phosphorylation results in cytoplasmic retention and higher level of linker phosphorylation causes nuclear localization.

The JNK phosphorylation sites in Smad2 and Smad3 have not been mapped. To unequivocally determine the effects of Smad2/3 linker phosphorylation on its nuclear accumulation, mutants of phosphorylation sites have to be built. These mutants can be used in further investigation of the influences of Smad2/3 linker phosphorylation on their subcellular localization.

Interestingly, when the endogenous JNK activity was blocked by overexpression of dominant-negative JNK^{APF} or C3 exotransferase, the nuclear accumulation of transfected Smad3 was abolished in Mv1Lu cells. This suggests that endogenous JNK activity is required for Smad3 nuclear translocation in response to TGF- β . Whether this is also true in other cell lines and whether this requirement depends on Smad3 linker phosphorylation remains to be determined.

The findings that Smad2/3 linker phosphorylation by JNK promoted their nuclear accumulation were not consistent with previous findings that Smad2/3 linker phosphorylation by ERK inhibited their nuclear localization. Several reasons can be speculated to account for this discrepancy. First, JNK may activate Smad independent pathways, such as AP-1 activation, that indirectly affect Smad subcellular localization. Second, JNK may phosphorylate a different subset of S/T-P motifs in the Smad2/3 linker region, and consequently exert distinct effects on nuclear localization. Third, the promotion of nuclear accumulation of Smad2/3 by linker phosphorylation may be a cell context dependent event that is only observed in some cell lines. To establish a causal link between linker phosphorylation and nuclear localization of Smad2/3, the JNK phosphorylation sites in Smad2/3 should be mapped, and the nuclear accumulation of the mutants should be examined.

HGF treatment induced the hetero-complex formation between endogenous Smad2/3 and Smad4 in RGM-1 cells. HGF treatment also induced the complex formation of overexpressed Smad4 and wild-type Smad2 or Smad3. Interestingly, HGF treatment also induced the interaction between Smad4 and C-tail mutated Smad2 or Smad3; whereas HGF treatment failed to induce the complex formation between Smad4 and Smad2/3 EPSM, in which the JNK phosphorylation sites were mutated. This suggests that HGF induces Smad2/3-Smad4 heteromerization through JNK-mediated phosphorylation of Smad2 or Smad3 linker region independent of the C-tail phosphorylation. The interaction induced by HGF between Smad2 and Smad4 was several folds weaker than that induced by TGF- β . In addition, overexpression of constitutively active MEKK-1 induced the association of overexpressed Smad2 and Smad4 in bovine aortic endothelial cells (BAECs). The induction of R-Smad-Smad4 association through linker phosphorylation was not observed in COS-1 or 293T cells. The interaction of Smad2/3-Smad4 may be cell context dependent.

Interestingly, MEKK-1 overexpression in bovine aortic endothelial cells (BAECs) induced the interaction of the co-activator p300 with Smad2. Taken together with the findings that linker phosphorylation promoted Smad2/3 nuclear localization and hetero-complex formation with Smad4, the findings suggest that MEKK-1 and presumably other JNK activators, can lead to the activation of Smad transcription independent of the C-tail phosphorylation in certain cell lines.

Consistent with the Smad2/3 nuclear accumulation, heteromerization with Smad4, and assocation of Smad2 with p300 induced overexpression of MEKK-1, MEKK-1 overexpression induced the activity of PAI-1 promoter, 3TP-Luc reporter, and GAL4-Smad2*C, which contained part of the linker region and the entire C-domain of Smad2 and had the C-terminal motif deleted and fused to the DNA binding domain of GAL4. Similarly, consistent with JNK promotion of Smad2 nuclear accumulation, co-

overexpression of JNK with Smad3 in Mv1Lu cells significantly increased the activity of (CAGA)₁₂-Luc (a Smad3 specific TGF-β responsive reporter) irrespective of the Cterminus of Smad3. In HSCs or RGM-1 cells, treatment with PDGF or HGF activated JNK. Over-expression of Smad3 activated the PAI-1 promoter reporter PF1-Luc. Consistent with the HGF-induced Smad3 nuclear accumulation and Smad3-Smad4 association, treatment of cells with PDGF or HGF increased the activity of wild-type Smad3 as well as Smad3 3SA (in which C-terminus was mutated) on PF1-Luc. Remarkably, the ability of Smad3 EPSM (in which the putative JNK sites were mutated) to activate PF1-Luc was not further increased by PDGF or HGF treatment. This suggested that HGF or PDGF increased the activity of Smad3 on the PAI-1 promoter through JNK-mediated linker phosphorylation, independent of the C-terminus, and that linker phosphorylation of Smad3 was sufficient to induce PAI-1 promoter activity.

It is worth pointing out that JNK activation induces c-Jun phosphorylation and the activation of AP-1 transcription factor. AP-1 transcription factor is known to enhance Smad activity in several promoter contexts, including 3TP-Luc and GAL4-Smad2/3 fusion. Thus, the positive effects of JNK overexpression or HGF treatment on TGF- β /Smad target genes may included some non-specific effects through AP-1 activation.

The effects of JNK on Smad3 are promoter specific. Although HGF increased PAI-1 promoter activity, it decreased the CDK inhibitor p15INK4B promoter activity. Smad3 C-tail phosphorylation, but not linker phosphorylation, stimulated p15 promoter activity, whereas both linker and C-tail phosphorylation of Smad3 contributed to the activation of PAI-1 promoter. Since linker phosphorylation of Smad3 decreased C-tail
phosphorylation, HGF treatment decreased p15 promoter activity through the decrease of Smad3 C-tail phosphorylation.

Plasminogen Activator Inhibitor-1 (PAI-1) is an important modulator of extracellular matrix and cell migration. It conducts the cells to migration and invasion by blocking cellular adhesion and by promoting basement membrane degradation. In a model of acue liver injury by CCl₄ administration, the PDGF and TGF- β in the damaged area promoted the migration of HSCs (Hepatic Stellate Cells) from the space of Disse to the damaged region. In the development of colorectal carcinoma, the linker region phoosphorylation of smad3 increased whereas the C-tail phosphorylation of Smad3 decreased. Decreased C-tail phosphorylation may cause loss of induction of CDK inhibitors such as p15 by TGF- β , whereas increased linker phosphorylation specifically activated genes that promote migration and invasion such as PAI-1. Therefore, the change in Smad3 phosphorylation sites may underlie the switch of TGF- β from a tumor suppressor to a promoter of tumor invasion and metastasis. However, this hypothesis remains to be verified by more experiments.

1.5.4 p38 MAPK

PET¹⁷⁹P, AGS²⁰⁴P, NLS²⁰⁸P, and PMS²¹³P in Smad3 and PET²²⁰P, TGS²⁴⁵P, ELS²⁵⁰P, and TLS²⁵⁵P in Smad2 are (P/x)-x-(S/T)-P sites that are potential targets for proline-directed protein kinases that include the MAPK superfamily. p38 MAPKs belong to the MAPK superfamily. The (P/x)-x-(S/T)-P motifs in Smad2/3 linker region are potential phosphorylation sites for p38 MAPK. p38 MAPKs are encoded by four

genes, p38 α , p38 β , p38 γ , and p38 δ . The specific inhibitor SB203580 inhibits p38 α and p38 β , but does not inhibit p38 γ and p38 δ .

In various cell lines, such as RGM-1 (normal rat gastric epithelial cell line), Mv1Lu (Mv1Lu lung epithelial cell line), HaCaT (human epidermal keratinocytes), and PC3-M (a metastatic prostate cancer cell line), TGF- β induces slow but sustained p38 activation that last for several hours. p38 activation can occur both in a Smad-independent manner (e.g. in mouse mammary epithelial cells NMuMG) and in a Smad dependent manner (e.g. in pancreatic cancer cells Panc-1).

p38 was found to phosphorylate Smad1 and Smad3 *in vivo* or *in vitro*. When HaCaT cells were treated with UV or hypertonic NaCl that stimulate JNK and p38 activity, the endogenous Smad1 was found to be phosphorylated on the PxSP motifs [45]. Notably, pretreatment of cells with two inhibitors SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor) abolished UV induced Smad1 linker phosphorylation, suggesting that JNK and p38 phosphorylated Smad1 linker region in response to UV stress.

Activated p38 that is immunoprecipitated from cell lysates phosphorylates recombinant GST-Smad3 but not GST-Smad2 *in vitro* [146]. In myofibroblasts (MFBs), p38 activated by TGF- β phosphorylated Smad3 on S208/S213 sites [148]. The activation of p38 by TGF- β was significantly inhibited by the p38 inhibitors PD169316 or SB203580. The TGF- β induced Smad3 linker phosphorylation was strongly inhibited by PD169316 or SB203580 in a dose dependent manner. The inhibition of Smad3 linker phosphorylation by PD169316 and SB203580 closely correlated with the inhibition of p38 activation by PD169316 and SB203580. The findings strongly suggested that in MFBs, TGF- β activated p38 phosphorylated Smad3 linker region. Phosphorylation of

Smad3 linker region does not require its C-tail phosphorylation, since Smad3 3SA can also be phosphorylated by p38 in response to TGF- β . In a separate study utilizing the human breast cancer cell line MCF10CA1h cells, p38 activated by TGF- β treatment mainly phosphorylated two S/T-P motifs, S²⁰⁴P and S²⁰⁸P [149]. The TGF- β induced phosphorylation on S204 and S208 was significantly inhibited by the p38 inhibitor SB203580. The p38 phosphorylation sites in the Smad3 linker region were reported differently in different studies (S208/S213 vs. S204 & S208). The exact p38 phosphorylation sites may be dependent on different cellular contexts.

In metastatic prostate cancer cells PC3-M, inhibition of endogenous p38 activity partially blocked TGF- β induced Smad3 nuclear accumulation, whereas SB203580 only had minimal effect on the nuclear translocation of Smad2 [146]. This finding is consistent with the fact that Smad2 cannot be phosphorylated by p38 in vitro. In PC3-M, p38 activity was required for Smad3 nuclear translocation. In human gingival fibroblasts, co-expression of constitutively active MKK3b, an upstream activator of p38, with Smad3 resulted in Smad3 nuclear localization in the absence of TGF- β [147]. Thus, in human gingival fibroblasts, activation of endogenous or transfected p38 was sufficient for Smad3 nuclear accumulation. In myofibroblasts (MFBs), immunostaining with a phospho-peptide antibody recognizing phosphorylated Smad3 S208/S213 sites revealed exclusive nuclear staining, suggesting that Smad3 linker phosphorylation by p38 promoted Smad3 nuclear accumulation. The findings strongly indicated a positive role of p38 in Smad3 nuclear accumulation induced by TGF-B. Whether p38 promoted Smad3 nuclear accumulation through linker phosphorylation remains to be investigated. For this purpose, the Smad3 mutants of p38 phosphorylation sites may be used.

The promotion of nuclear accumulation of Smad3 by p38 was in contrast with inhibition of Smad3 nuclear accumulation by ERK through linker phosphorylation. It is possible that in addition to phosphorylating Smad3 llinker region, p38 also activate a Smad-independent pathway to promote Smad3 nuclear accumulation. Alternatively, p38 may only target a subset of the (P/x)-x-(S/T)-P motifs that upon phosphorylation, exerts positive effect on Smad3 nuclear localization.

In myofibroblasts (MFBs), TGF- β treatment induced Smad3 linker phosphorylation, but not Smad3 C-tail phosphorylation. Smad3 linker phosphorylation induced by TGF- β is mediated by TGF- β activated p38 MAPK. Smad3 linker phosphorylation induced the complex formation between endogenous Smad3 and Smad4. It is not known whether Smad3 linker phosphorylation in other cell lines also induces Smad3-Smad4 association. Electrophoretic Mobility Shift Assay (EMSA) also revealed that in MFBs, linker phosphoryated Smad3, but not C-tail phosphorylated Smad3, formed a complex with PAI-1 romoter DNA. Taken together with p38 induced Smad3 nuclear translocation and Smad3-Smad4 complex formation, the finding that linker phosphorylated Smad3 also occupy promoter DNA suggested that TGF- β can activate Smad3 transcription through p38 mediated linker phosphorylation.

Phosphorylation of Smad3 by p38 had distinct effects in different cellular contexts. In metastatic prostate cancer cells PC3-M, Smad3 and p38 are both required for TGF- β induced cell adhesion. p38 was required for Smad3 nuclear translocation possibly through Smad3 phosphorylation. Thus, Smad3 phosphorylation by p38 and nuclear translocation may be an essential step in TGF- β mediated cell adhesion.

p38 and Smad3 cooperated to activate the expression of collagenase-3 in human gingival fibroblast [147]. Activation of p38 by overexpression of MKK3b caused Smad3 nuclear translocation in the absence of TGF- β . Thus, p38 induced Smad3 nuclear localization may be an important mechanism of crosstalk between p38 pathway and Smad pathway in their cooperation to activate collagenase-3 expression.

In a chronic liver injury model, hepatic stellate cells (HSCs) transdifferentiate into myofibroblasts (MFBs). Myofibroblasts plays a prominent role in liver fibrogenesis in chronic liver injury. Interestingly, in myofibroblasts (MFBs), the linker region of Smad3, but not the C-tail, was phosphorylated [148]. Autocrine TGF- β signaling activated p38 mediated Smad3 linker phosphorylation. Linker phosphorylated Smad3 translocated into the nucleus, formed complex with Smad4, and bound to PAI-1 promoter DNA. Linker phosphorylated Smad3 was essential in activating the expression of genes involved in fibrogenesis, such as collagen $\alpha 2(I)$ and PAI-1.

Finally, in the human breast cancer cell line MCF10A1h, TGF- β activated p38, which in turn phosphorylated Smad3 on S204 and S208 [149]. Mutation of the p38 phosphorylation sites, as well as pretreatment of cells with p38 inhibitor SB203580, strongly inhibited activity of Smad3 on the (CAGA)₁₂-Luc and the (ARE)₃-Luc reporters. Importantly, p38 inhibition partially blocked TGF- β induced cell cycle inhibition. Therefore, in certain cells, p38 activated by TGF- β may enhance TGF- β responsive gene activation and growth inhibition through phosphorylating two S-P motifs in the Smad3 linker region.

1.6 Regulation of Smad activity by dephosphorylation of phosphorylated (S/T)-P motifs in their linker region and MH1 domain

(P-x-)(S/T)-P motifs in the linker region and MH1 domain of Smad1, 2, and 3 are targets for multiple proline-directed kinases. Phosphorylation of the (S/T)-P motifs on Smad1, 2 and 3 represses their activity through inhibition of nuclear accumulation. When certain subsets of (S/T)-P motifs of Smad2/3 are phosphorylated by ERK, JNK or p38, Smad2/3 activity could also be elevated, through promotion of nuclear translocation, induction of R-Smad-Smad4 complex formation, or protein stabilization. The removal of phosphate groups from serine or threonine is the reverse process of phosphorylation. The phosphorylation status of linker region and the activity of Smad1, 2 and 3 are under the joint control of kinases and phosphatases. SCP1/2/3 were found to be the unique phosphatases for the Smad1/2/3 linker region (Table 1.8) [94, 98].

In one study to search for potential phosphatase(s) for the (P-x-)(S/T)-P motifs in the MH1 domain and linker region of Smad2 and 3 (these motifs are: Smad2/3 T8, Smad2/3 T220/T179, Smad2 S245/S250/S255, Smad3 S204, Smad3 S208, Smad3 S213), the catalytic subunits of 40 protein serine/threonine phosphatases (P(S/T)Ps) and dual specificity phosphatases (DUSPs) were individually co-expressed with Smad2 or Smad3 in HEK293T cells, and the phosphorylation on Smad2/3 were monitored by phosphosphospecific antibodies against these (P-x-)(S/T)-P motifs of Smad2/3 [98]. In the absence of phosphatases, Smad2 and Smad3 were constitutively phosphorylated when over-expressed in 293T cells. Among all 40 phosphatases, only the expression of SCP1/2/3 (Small RNA polymerase II Carboxyl-terminal domain (CTD) Phosphatases) abolished the phosphorylation on the (P-x-)(S/T)-P motifs of Smad2/3. Interestingly, SCP1/2/3

reduce the phosphorylation of Smad2/3 T8, Smad2 S245/S250/S255, and Smad3 S204/S208/S213, but does not affect Smad2/3 T220/T179 phosphorylation.

In an independent study, SCPs were found to dephosphorylate the phosphorylated Ctail of Smad1 but not that of Smad2 [94]. The constitutive interaction of Smad1 and 2 with SCPs prompted the authors to examine whether SCPs also dephosphorylate the linker region of Smad1 and 2. Using antibodies against phosphorylated Smad1 S187, S195, and S206 respectively, and antibodies against Smad2 phosphorylated on S245/S250/S255, SCPs were shown to efficiently dephosphorylate Smad1/2 linker when over-expressed in 293 cells and the *Xenopus* embryos.

SCP1/2/3 belong to the FCP1/SCP family of phosphatases. Another two members of this family, FCP1 and SCP4/ CTDSL2, cannot dephosphorylate Smad2/3 linker region, despite their homology to SCP1/2/3. Thus, although both FCP1 and SCP1/2/3 act on phosphorylated serine 2 and serine 5 of the RNA polymerase carboxyl-terminal domain (CTD), they have differential specificity toward Smad2/3 linker region.

As expected, knockdown of endogenous SCPs in HaCaT cells increases the phosphorylation of the linker region of Smad1, 2, and 3. Consistent with the negative effects of linker phosphorylation on the activity of Smad2/3, knock down of SCPs in HaCaT cells dramatically reduced the response of endogenous Smad target genes Smad7, p21Cip1, p15INK4B, and JAGGED1 to TGF- β treatment, accompanied by a significant increase in Smad2/3 linker phosphorylation. On the contrary, overexpression of SCPs in HaCaT cells increased both the basal and TGF- β induced activities of the reporter genes (SBE)₄-Luc, p15-Luc, and p21-Luc.

SCPs only dephosphorylate the linker region of Smad2/3 but do not dephosphorylate the C-tail of Smad2/3. On the contrary, SCPs dephosphorylate both the linker and the C-tail of Smad1. Thus, knockdown of SCPs increases not only the inhibitory linker phosphorylation of Smad1, but also the activating C-tail phosphorylation of Smad1. Since the C-tail phosphorylation dominates over the linker phosphorylation in determining Smad1 activity, the net effect of SCP knockdown on Smad1 target gene expression is activation. As expected, SCP1/2 depletion by siRNA (small interfering RNA) increased both the basal and the BMP induced expression of the BMP/Smad1 target gene Id1. In summary, SCPs dephosphorylate Smad2/3 linker region but leave the phosphorylated C-tail domain intact leading to increased transcriptional activities, whereas Smad1 is dephosphorylated on both the C-tail and the linker region by SCPs, and is reset to the basal state as a result.

Many protein phosphatases associate with regulatory subunits to obtain target selectivity. Although SCPs dephosphorylates both the linker and the C-tail of Smad1, the requirement for regulatory proteins may differ. When overexpressed in 293T cells, only the linker region is efficiently dephosphorylated by SCPs, whereas the C-tail phosphorylation is barely affected even in the presence of overexpressed SCPs. Dephosphorylation of Smad1 C-tail by SCPs may require additional endogenous rate-limiting factors. Overexpression of inactive SCP mutants in 293T cells not only failed to decrease Smad1 C-tail phosphorylation, but even increased C-tail phosphorylation. This is most likely the result of titration of essential endogenous factors by the overexpressed dominant negative SCPs, and the consequent inhibition of endogenous SCP activity. Interestingly, SCPs overexpressed in *Xenopus* embryos can efficiently dephosphorylate

Smad1 C-tail, suggesting that the rate-limiting factor in mammalian cells may be abundantly present in *Xenopus* embryos.

On the contrary, the dephosphorylation of linker region of Smads by SCPs does not require additional factors. In a cell free *in vitro* dephosphorylatoiun assay, recombinant SCPs can dephosphorylate recombinant Smad2/3 that is phosphorylated *in vitro* by ERK.

Whether additional phosphatases for Smad linker region is an open question. The screening by overexpressing the catalytic subunits of 40 phosphatases may miss out potential candidates that require additional regulatory proteins to dephosphorylate the linker region. In addition, there are many dual specificity protein phosphatases (DUSPs) that are not included in the screen. Indeed, none of the 40 phosphatases dephosphorylate the Smad2/3 T220/T179 site. Thus, the phosphatase for T220/T179 site may exist in the DUSP category that remains to be screened.

What are the mechanisms that SCPs increase the transcriptional activities of Smad2/3? SCPs catalyze the removal of inhibitory phosphates from the Smad2/3 linker region. An interesting observation is that when SCPs were over-expressed in 293T cells, the TGF- β induced C-tail phosphorylation of Smad2 or Smad3 was increased. This suggests that under normal conditions, linker phosphorylation of Smad2/3 may inhibit their C-tail phosphorylation. When Smad3 was co-transfected into Hela cells with SCP1, the Smad3 was localized to nucleus even in the absence of TGF- β . The nuclear localization of Smad3 in the presence of SCP1 overexpression could be a result of Smad3-SCP interaction and the fact that SCP1 is a nuclear protein. Thus, the SCP1 protein may bring the Smad3 protein into the nucleus through their interaction.

Alternatively, it has been demonstrated that linker phosphorylation of Smad2/3 inhibits their nuclear accumulation. The removal of phosphates by SCP1 from Smad3 may consequently promote Smad3 nuclear accumulation.

In summary, the activity of R-Smad is under the dynamic control of C-tail kinases (i.e. the type I receptors), C-tail phosphatases, linker kinases, and linker phosphatases.

1.7 Regulation of Smad activity through phosphorylation by non-proline directed kinases

Smad activity is also under the negative or positive control of several kinases that do not belong to the proline-directed protein kinases. They target selected serines or threonines that are not followed by prolines. Up to now, non-proline directed kinases that affect Smad activities include protein kinase C (PKC), Ca²⁺/calmodulin-dependent protein kinase II (CamKII), G-protein coupled receptor kinase -2 (GRK2), and casein kinase Iɛ (CKIɛ) (see Table 1.10 for a summary) [150-153].

1.7.1 Protein Kinase C (PKC)

Treating Mv1Lu cells (mink lung epithelial cell line, highly responsive to TGF- β) with phorbol 12-myristate 13-acetate (PMA, also known as phorbol easter), a strong protein kinase C (PKC) activator, resulted in the appearance of novel tryptic phospho- peptides of endogenous Smad2 and Smad3 [150]. This suggests that Smad2 and Smad3 may be a substrate of PKC. The appearance of additional phosphopeptides upon PMA treatment was efficiently inhibited by PKC inhibitors calphostin C and staurosporine, but not inhibited by MEK (upstream activator of ERK) inhibitor PD98059

Kinase Smad	Phosphory- lation sites	Cell Type	Effects of phosphorylation Ref	erences
PKC Smad2	S47, S110	Mv1Lu COS-1	no effect on the ability of Smad2 to activate (ARE) ₃ -Luc reporter	[150]
Smad3	S37, S70	Mv1Lu COS-1	abolished Smad3's DNA binding ability	[150]
<i>CamKII</i> Smad2, Smad3, Smad4,	Smad2:S110, S240, S260 not mapped for Smad3/4	COS-1 293T	abolished Smad2's nuclear accu- mulation in response to TGF-β, abolished Smad2-Smad3 hetero- merization	[151]
<i>GRK-2</i> Smad2	T197	HuH7 HepG2 CHO	phosphorylation of Smad2 by GRK- 2 inhibited TGF-β induced Smad2 C-tail phosphorylation	[152]
CKIE Smad1, Smad2, Smad3, Smad5	not mapped	HaCaT HepG2	CKIe phosphorylation of Smad2/3 activated their transcription activity CKIe decrease Smad3 level in a kinase activity-independent manner	[51, 153]

Table 1.10 Smad phosphorylation by non-proline directed kinases

Abbreviations are: PKC, protein <u>kinase</u> <u>C</u>; CamKII, Ca²⁺/<u>Calm</u>odulin-dependent <u>kinase</u> <u>II</u>; GRK-2, <u>G</u>-protein coupled receptor <u>kinase-2</u>; CKI ϵ , <u>casein kinase I ϵ ; ARE, <u>activin</u> response <u>e</u>lement</u>

Thus, the PMA induced Smad2/3 phosphorylation is due to the activation of PKC but not the MEK/ERK pathway. Additionally, stimulation of Mv1Lu cells with fetal bovine serum also led to the appearance of the same phosphopeptides characteristic of PMA treatment. Inhibitors of PLC γ and PKC, but not inhibitors of phosphatidylinositol-3kinase (PI3K) or MEK, blocked the appearance of these phosphopeptides.

Recombinant Smad2/3 can be phosphorylated *in vitro* by the PKC catalytic subunit, and tryptic digestion of Smad2/3 phosphorylated *in vitro* by PKC gave same tryptic phosphopeptides as those observed for endogenous Smad2/3 phosphorylated *in vivo* by PMA treatment. These observations suggest that Smad2 and Smad3 can be phosphorylated by PKC both *in vivo* and *in vitro* on the same sites.

To map PKC phosphorylation sites in Smad2/3, ³²P-labeled phosphopeptides were subjected to Edman degradation and phosphoamino acid analysis. The results from these assays indicate that serine 37 and serine 70 in Smad3, and the homologous sites serine 40 and serine 110 in Smad2, are phosphorylated by PKC. Phosphopeptide mapping of mutant Smad2/3 in which the putative PKC sites are replaced by alanines confirmed the finding that S37/S70 of Smad3 and S40/S110 of Smad2 are phosphorylated by PKC.

The PKC sites in Smad2/3 are within their MH1 domain. Mapping of Smad3 PKC sites onto the three dimensional structure of MH1 domain revealed that both S37 and S70 are located to a region that faces the DNA. Since phosphorylation introduces negative phosphate groups onto the targeted serine residues, PKC phosphorylation may abrogate the binding of Smad3 MH1 domain to the negatively charged DNA phosphate backbone. Pre-phosphorylation of GST (Glutathione-S-Transferase) tagged Smad3 MH1

domain by PKC indeed blocked DNA binding by Smad3 MH1 domain in EMSA (Electrophoretic Mobility Shift Assay) analysis.

Consistent with the finding that PKC phosphorylation of Smad3 abolishes its DNA binding activity, treatment of cells with PMA inhibited TGF- β induced activation of the (CAGA)₁₂-Luc reporter. When the PKC target residues (S37 and S70) in Smad3 are replaced by aspartic acids (D) that mimick phosphorylation, the DNA binding activities of the mutants were severely impaired. Interestingly, substitution of S70 with alanine also reduced DNA binding, highlighting the importance of a serine residue at 70 position for Smad3 DNA binding. All three DNA binding mutants of Smad3, S37D, S70D, and S70A, are defective in activating the TGF- β responsive reporter gene (CAGA)₁₂-Luc.

Unlike Smad3, Smad2 MH1 domain cannot bind to DNA. Phosphorylation of Smad2 by PKC, or substitution of S40/S110 by acidic residues, does not affect the activity of Smad2 on the luciferase reporter gene (ARE)₃-Luc. Activation of (ARE)₃-Luc by Smad2 does not require DNA binding by Smad2, but involves the interaction of Smad2 MH2 domain with Fast-1. This observation suggests that although PKC phosphorylation inhibits Smad3 DNA binding activity, it does not affect transactivation by Smad2/3.

When cells were stably transfected with wild-type Smad3 and treated with a combination of TGF- β and PMA, cells start to overgrow, but they do not form foci. Notably, cells stably transfected with the DNA binding defective S37D, S70D, or S70A mutant Smad3 respond to TGF- β +PMA treatment with foci formation. The DNA binding deficient Smad3 showed increased ability to cause transformation of cells than wild-type Smad3. PMA treatment also abolished TGF- β induced apoptosis in Mv1Lu cells, probably through inactivating Smad3's DNA binding ability. Thus, PKC

phosphorylation of Smad3 and the selective elimination of Smad3's DNA binding activity re-directs the function of Smad3 from apoptosis induction to promoting transformation.

1.7.2 Ca²⁺-calmudulin-dependent protein kinase II (CamKII)

To study the potential effects of cellular calcium signaling on TGF- β response, the compound thapsigargin was used [151]. Thapsigargin inhibits Ca²⁺-ATP pump and raises intracellular Ca²⁺ concentration. In COS-1 cells, thapsigargin pre-treatment abolished the TGF- β induced expression of the endogenous PAI-1 gene. When the TGF- β responsive reporters 3TP-Luc (which contains the TGF- β response element in PAI-1 and collagen type I promoters) and (ARE)₃-Luc were transfected into cells, their induction by TGF- β was completely abolished by thapsigargin pre-treatment. Thus, increased intracellular Ca²⁺ strongly inhibits TGF- β signaling.

One important mediator of biological effects of Ca^{2+} concentration changes is the Ca^{2+} -Calmodulin dependent protein kinase II (CamKII), whose activity is upregulated by Ca^{2+} -Calmodulin binding. To investigate the role of CamKII in Ca^{2+} mediated inhibition of TGF- β signaling, a specific inhibitor of CamKII, KN-93, was used. In the presence of KN-93, Ca^{2+} elevation cannot inhibit TGF- β signaling. Expression of a truncated, constitutively active CamKII (CamKII₁₋₂₉₀) mimicked the effect of Ca²⁺ concetration increase on TGF- β signaling. Thus, Ca^{2+} signaling inhibits TGF- β signaling through CamKII.

In Smad2, there are one Rxx(S/T) motif and four SxD motifs, which are potential phosphorylation sites for CamKII. The <u>RCSSMS</u> motif is at the C-terminus, whereas

three SxD motifs are clustered in the linker region and one SxD was located in MH1 domain. Smad2 was abundantly phosphorylated by CamKII *in vitro*. When shorter peptides each containing only one potential CamKII site were tested in *in vitro* kinase assay by CamKII, only three sites (S¹¹⁰LD, S²⁴⁰MD, and S²⁶⁰LD) were phosphorylated, whereas the other two (S²²⁷ED, RCSS⁴⁶⁵MS) were not.

When Smad2, 3 and 4 were co-expressed with CamKII₁₋₂₉₀, all three proteins can be phosphorylated, whereas the Smad2 mutant in which the three CamKII sites were mutated was only minimally phosphorylated. CamKII induced Smad3 phosphorylation was significantly weaker than Smad2 phosphorylation. Two of the three CamKII sites in Smad2 are conserved in Smad3, and they are S⁷⁰LD, and S¹⁹⁹MD. It remains to be shown whether they are phosphorylated *in vivo* by CamKII. Interestingly, S70/S110 in Smad3/2 is also phosphorylated by PKC. If Smad3 is indeed phosphorylated at S70 by CamKII, the phosphorylation will lead to loss of DNA binding.

A phosphopeptide antibody was successfully raised against phosphorylated S240 site in Smad2. This antibody only recognizes Smad2 phosphorylated on S240. Multiple signals including EGF, PDGF, and TGF- β can induce the phosphorylation of Smad2 on S240 site in 293T cells and in HepG2 cells. Thus, CamKII integrates signals from multiple cellular pathways and converts the signals into phosphorylation status change of Smad2.

Overexpression of GFP-Smad2 with CamKII₁₋₂₉₀ blocked the TGF- β induced Smad2 nuclear localization in COS-1 cells. Notably, GFP-Smad2 (CamKII sites mutant) still translocates to nucleus in response to TGF- β even when the constitutively active CamKII₁₋₂₉₀ was co-expressed. CamKII phosphorylation does not seem to change the TGF- β induced C-tail phosphorylation of Smad2.

Interestingly, CamKII overepression induced Smad2-Smad4 heterocomplex formation in the absence of C-tail phosphorylation. CamKII also induce the complex formation between Smad4 and CamKII site mutant Smad2. Thus, the complex formation of Smad2 and Smad4 is independent of phosphorylation by CamKII. Most likely, the CamKII induced Smad2-Smad4 association is non-specific effect of CamKII overexpression.

On the contrary, CamKII abolished the heteromerization of Smad2 and Smad3 in response to TGF- β . CamKII expression does not abolish the interaction between Smad3 and Smad2 mutated on the CamKII sites. Thus, CamKII mediated blocking of Smad2/3 complex formation is dependent on Smad2 phosphorylation by CamKII.

The mechanisms underlying the inhibition of Smad2 nuclear accumulation and disruption of Smad2/Smad3 complex mediated by CamKII phosphorylation of Smad2 is unknown. Since TGF- β also induces CamKII activation and Smad2 S240 phosphorylation, TGF- β mediated Smad2 phosphorylation by CamKII represses Smad2 activity through negative feedback inhibition.

1.7.3 G-protein coupled receptor kinase 2 (GRK2)

G-coupled receptor kinase 2 (GRK2), a kinase involved in desensitization of Gprotein coupled receptors (GPCRs), is identified as a TGF- β /activin target gene in a microarray based screen [152]. The GRK family includes several other members such as GRK 3, 4, 5, and 6. Among these GRKs, only GRK2 is induced by TGF- β signaling. GRKs have highly homologous kinase domains. Overexpression of GRK2, or any other GRK, strongly repressed TGF- β induced 3TP-Luc activity. The kinase defective GRK2 does not repress 3TP-Luc activity, suggesting that the kinase activity of GRK2 is required for inhibition of TGF- β signaling.

When the endogenous GRK2 was depleted by siRNA in the liver cancer cell line HuH7, TGF- β induced a more robust induction of the CDK inhibitor p15 and the proapoptotic protein Bax. In addition, the repression of c-myc in GRK2 depleted cells was more complete than in control cells. These observations suggest that endogenous GRK2 repress the cytostatic and pro-apoptotic functions of TGF- β in liver cancer cells.

Depletion of GRK2 increased TGF- β induced Smad2 C-tail phosphorylation, whereas overexpression of GRK2 diminished Smad2 C-tail phosphorylation. A kinase dead GRK2 does not decrease Smad2 C-tail phosphorylation. This represents an important mechanism that GRK2 inhibits TGF- β signaling. Consequently, GRK2 overexpression abolished TGF- β induced Smad3 nuclear accumulation.

The finding that GRK2 represses TGF- β signaling in a kinase activity dependent manner prompted the anthors to examine the possibility that GRK2 may directly phosphorylate Smad2/3. The MH1 and MH2 domains of Smad2/3 directly interact with GRK2. In contrast, only the linker region of Smad2, but not the MH1 or MH2 domains is phosphorylated by GRK2 *in vitro*. To map the GRK2 phosphorylation sites in Smad2, serines or threonines in the Smad2 linker region were mutated individually or in combination. The mutation of T197 in Smad2 strongly inhibited GRK2 mediated Smad2 phosphorylates Smad2 on a single site in the linker region: T197.

How does the linker phosphorylation of Smad2 inhibit C-tail phosphorylation? In an *in vitro* kinase assay, Smad2 or Smad3 pre-phosphorylated in the linker region can no longer be phosphorylated by constitutively active type I receptor. The linker phosphorylation of Smad2/3 may change the conformation of the molecule that they can no longer be recognized by the type I receptor.

The regulation of Smads by GRK2 and the induction of GRK2 by TGF- β /activin provides a negative feedback control mechanism in TGF- β /Smad signaling. In addition, GRK2 can also be induced by other stimuli such as norepinephrine (NE), an agonist of the GPCR (G-protein coupled receptor) α_1 -adrenergic receptor (α_1 -AR). Thus, GRK2 can integrate signals from multiple cellular pathways and feed them into the Smad signaling.

1.7.4 Casein Kinase I ε (CKI ε)

Casein Kinase I ε (CKI ε) was identified as a protein that interacts with both Smads and TGF- β type I and type II receptors [153]. CKI ε interacts strongly with T β RI and T β RII. The interaction was constitutive irrespective of ligand stimulation. In contrast, interaction of CKI ε with Smad1, 2, and 3 is weaker than interaction with receptors, occurs in the absence of TGF- β , and is transiently disrupted by TGF- β treatment. Smad2/3 re-associates with CKI ε after 4 hours of TGF- β stimulation.

Smad1, 2, 3, and 5 are phosphorylated by CKI ϵ , and the phosphorylation sites in Smad3 was mapped to MH1 domain and linker region. When CKI ϵ is co-expressed with the TGF- β responsive reporter (SBE)₄-Luc, it repressed the basal activity, whereas stimulated the TGF- β induced activity. The kinase inactive CKI ϵ repressed the TGF- β induced activity of $(SBE)_4$ -Luc. This suggests that CKI ϵ phosphorylation of Smad3 increased the activity of Smad3 when the C-tail is also phosphorylated. Consistent with this hypothesis, inhibition of kinase activity of endogenous CKI ϵ by the chemical inhibitor IC261 strongly inhibited the TGF- β activated (SBE)₄-Luc reporter.

Surprisingly, when endogenous CKI ϵ protein was knocked down by RNAi, both the basal and TGF- β induced activities of (SBE)₄-Luc were significantly increased. Accompanied with the increase in reporter activity, the protein level of endogenous Smad3 was significanlly elevated. The inhibition of CKI ϵ kinase activity does not result in Smad3 protein level increase. This suggests that CKI ϵ plays a distinct role in controlling Smad3 protein stability, and that control of Smad3 level by CKI ϵ is independent of CKI ϵ 's kinase activity. Consistent with this proposal, overexpression of either the wild-type CKI ϵ or the kinase-dead CKI ϵ leads to decreased Smad3 protein level. Consequently, overexpression of CKI ϵ protein decreased the basal activity of (SBE)₄-Luc reporter, regardless of whether it possess kinase activity or not.

In summary, CKI ϵ palys dual roles in regulating TGF- β signaling. CKI ϵ can directly phosphorylate Smad3 and activate activity of Smad3; at the same time, CKI ϵ reduces the stability and steady state level of Smad3 in a kinase activity-independent manner.

The exact phosphorylation sites of CKIE in Smad3 have not been mapped. In addition, the mechanism by which CKIE phosphorylation activates Smad3 transcriptional activity remains to be determined.

Chapter II: Identification and Characterization of ERK MAP Kinase Phosphorylation Sites in Smad3

2.1 ABSTRACT

Smad3 is phosphorylated by ERK MAP kinase upon EGF treatment. We have mapped the ERK phosphorylation sites to Ser 208, Ser 204 and Thr 179 in Smad3. We show that upon EGF treatment, Smad3 is rapidly phosphorylated in these sites, peaking at ~15-30 minutes, and that MEK1 inhibitors PD98059 and U0216 inhibit Smad3 phosphorylation induced by EGF. Ser 208 is the best ERK site in Smad3. Its phosphorylation shows the highest EGF induction in Smad3. It is also a very sensitive site to EGF treatment, significantly responding to low concentrations of EGF. These three sites are also phosphorylated by recombinant ERK2 *in vitro*. We have compared the kinetic parameters of Smad3 with those of ELK1 and MBP for ERK2. We further show that mutation of the ERK phosphorylation sites increases the ability of Smad3 to stimulate a Smad target gene, suggesting that ERK phosphorylation inhibits Smad3 activity.

2.2 INTRODUCTION

TGF-β family members regulate a wide variety of biological responses [155]. Smad proteins can transduce the TGF-β signal at the cell surface into gene regulation in the nucleus [1, 4, 156-158, 170]. The Smad family is divided into different groups [1, 4, 156-158, 170]. One group includes the pathway-specific Smads, also referred to as R-Smads, which are phosphorylated by receptor kinases. For example, Smad2 and Smad3 are phosphorylated by the TGF-β receptor kinase in the C-terminal tail. The second group consists of the Co-Smads, which are commonly used in various TGF-β family members signaling pathways. Smad4 is the only member of this group in vertebrates. Upon TGF-ß treatment, Smad2 and Smad3 are phosphorylated by the TGF-ß receptor, form complexes with Smad4 and together accumulate in the nucleus. Often in conjunction with DNA binding factors, Smads regulate transcription of responsive genes [1, 4, 156-158, 170].

Smad activity is also regulated by phosphorylation through non-receptor kinases. Smads contain a proline-rich region, termed the linker region, which connects the conserved N-terminal and C-terminal domains. The linker region is divergent in sequence and in length among Smads [1, 4, 156-158, 170]. The linker regions of R-Smads contain demonstrated as well as suspected phosphorylation sites for prolinedirected kinases, such as ERK MAP kinase, c-Jun N-terminal kinase, p38 MAPK, cyclin-dependent kinases, as well as other kinases, such as Ca2+-calmodulin-dependent kinase II [46-50, 132, 133, 138, 139, 144, 145, 148, 149, 151, 172-175]. For example, we have mapped the CDK phosphorylation sites to Thr 8, Thr 179 and Ser 213 in Smad3, and we have shown that CDK phosphorylation of Smad3 inhibits its transcriptional activity and antiproliferative function [50, 175]. There have been a number of reports on ERK phosphorylation and regulation of Smads, some of which appear to be in conflict with each other in the conclusions. The underlying mechanisms remain to be elucidated. Identification of the ERK phosphorylation sites in Smads is an essential step towards this goal.

Smad3 contains a total of nine potential ERK phosphorylation sites. Through mutational analysis, previous studies have shown that the ERK phosphorylation occurs within the four sites in the linker region. Mutation of these four sites dramatically reduces phosphorylation by ERK both *in vivo* and *in vitro* [48]. The four potential ERK

phosphorylation sites in the Smad3 linker region are: Thr 179 (T179), Ser 204 (S204), Ser 208 (S208), and Ser 213 (S213). We previously briefly described that in human HaCaT keratinocytes, ERK phosphorylated S204 and S208, and that T179 is phosphorylated more by CDK than by ERK [50]. In this study, we characterized ERK phosphorylation of Smad3 in epithelial cells, using Mv1Lu mink lung epithelial cells as a model system. We show that EGF treatment induces a rapid phosphorylation of S208, S204 and T179. Among the ERK phosphorylation sites in Smad3, S208 is the best site. Its phosphorylation is greatly induced by EGF treatment, and it is the most sensitive site in Smad3, responding to low doses of EGF. We further show that mutation of the ERK phosphorylation sites to non-phosphorylatable residues increases Smad activity to stimulate a TGF-B/Smad responsive gene, suggesting that ERK phosphorylation inhibits Smad3 activity.

2.3 MATERIALS AND METHODS

2.3.1 pS208, pS204, pT179 and pS213 phospho-peptide antibodies and other antibodies

Each of the phosphopeptide antibodies was raised in rabbits, affinity purified against the phosphopeptide antigen, and cross-absorbed against the unphosphorylated peptide of the same sequence. The specificities of each of these four phosphate-specific antibodies have been demonstrated by several analyses. First, each of the phosphopeptide antibodies recognizes only the wild type Smad3 but not the corresponding mutant Smad3 by immunoblotting (Supplementary Fig. 4a in reference 50). Second, each of the phosphopeptide antibodies can recognize overexpressed wild type Smad3 but not the corresponding mutant form in an immunoprecipitation assay (Supplementary Fig. 4b in reference 50). Third, treatment of the phosphorylated Smad3 with a phosphatase leads to the disappearance of the phosphorylated band (Supplementary Fig. 4c in reference 50). Fourth, the band recognized by each of the phosphopeptide antibodies is Smad3, as none of these antibodies can detect a band that comigrates with Smad3 using cell extracts from Smad3^{-/-} mouse embryonic fibroblasts (Supplementary Fig. 4d in reference 50). The antibodies against pERK, ATF3 and PCNA were purchased from Santa Cruz Biotechnology, Inc. The ERK antibody was from Signaling Solutions. The Smad3 antibody was from Zymed laboratories. The GAPDH antibody was from Ambion, Inc.

2.3.2 In vitro kinase assay

Recombinant activated ERK2 MAP kinase was purchased from Calbiochem. Myelin basic protein was purchased from Sigma. The kinase reaction contains the buffer (20 mM MOPS-Na, pH 7.4, 10 mM MgCl₂, 1 mM EGTA), 100 μ M ATP, 3 μ Ci [γ -³²P]ATP, and appropriate amount of substrate and kinase, in a volume of 30 μ l. The kinase reaction was performed at 30°C for 30 min or 20 min. The substrate titration experiments were performed under a condition that phosphate incorporation was in a linear range over the time. The reaction was terminated by addition of SDS protein gel sample buffer. Phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography. Non-radioactive kinase reactions for immunoblot analysis by phospho-peptide antibodies contained the buffer (20 mM MOPS-Na, pH 7.4, 10 mM MgCl₂, 1 mM EGTA), 0.5 mM ATP, 0.3 μ M GST-Smad3, and 26 ng activated ERK2 in 30 μ l. Reactions were carried out at 30°C for 30 min.

2.3.3 Immunoblot analysis

Immunoblot analyses were performed essentially as previously described [50, 169]. In brief, cells were lysed in the TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% NP-40) in the presence of protease and phosphatase inhibitors. 30 μ g cell lysates were then loaded on a gel and immunoblotted by appropriate antibodies. pS208, pS204, and pT179 antibodies were used at 0.15 μ g/ml. pS213 antibody was used at 0.6 μ g/ml.

2.3.4 Transfection and reporter gene assay

HepG2 cells in 60 mm dishes were transfected by DEAE-dextran and analyzed for luciferase activity as previously described [107]. Luciferase activities were normalized by the cotransfected *Renilla* luciferase control driven by pRL-TK (Promega). Results represent the mean and standard deviation of four independent transfection experiments.

2.3.5 Retroviral infection

Wild type and each of the Smad3 phosphorylation mutants were cloned into the pLZRSΔ-IRES-GFP retroviral vector [176]. The resulting retroviral plasmids were transfected into the ecotropic phoenix packaging cells to produce retroviruses as described previously [177]. Mouse C2C12 myoblasts were infected with greater than 95% efficiency. 72 hours post-infection, cells were split into fresh medium. Cells were then harvested and analyzed 24 hours later.

2.4 RESULTS

2.4.1 S208, S204, and T179 in Smad3 are phosphorylated by MAP kinase in response to EGF treatment

Previous studies have shown that MAP kinase phosphorylation of Smad3 occurs in the linker region, within the four sites: T179, S204, S208, and S213 [48]. We have generated phospho-peptide antibodies against each of these four sites [50]. The specificities of each of these four phosphate-specific antibodies have been demonstrated by several analyses, including immunoblot, immunoprecipitation, phosphatase treatment, and confirming that the recognized band is Smad3 by comparing wild type cells with Smad3 deficient cells (Supplementary Fig. 4 in reference 50).

To determine which of the four sites is phosphorylated by MAP kinase in epithelial cells, Mv1Lu mink lung epithelial cells were serum-starved overnight, treated with EGF (50 ng/ml) for 30 minutes and then analyzed for Smad3 phosphorylation at these sites. As shown in Fig 2.1A, EGF treatment led to a rapid phosphorylation of S208, S204 and T179. We have previously shown that CDK4 and CDK2 phosphorylate T8, T179 and S213 but not S204 or S208 *in vivo* [50]. Since the EGF treatment is only for 30 minutes, the EGF induced phosphorylation of T179 is not due to the activation of CDK.

S208, S204 and T179 can also be phosphorylated by MAP kinase *in vitro* (Fig 2.1B). Although the S213 site can also be phosphorylated by MAP kinase *in vitro* (Fig 2.1B), we detected very little or no EGF-induced phosphorylation of endogenous S213 *in vivo* in either Mv1Lu epithelial cells (Fig 2.1B) or in HaCaT cells [50].

2.4.2 S208 is the best MAP kinase phosphorylation site in Smad3.

While S208, S204, and T179 were all phosphorylated by MAP kinase in response to



Figure 2.1 S208, S204, and T179 in Smad3 are phosphorylated by ERK *in vivo* and *in vitro*

(A) S208, S204, and T179 in Smad3 are phosphorylated in response to EGF treatment. Mv1Lu cells were serum-starved overnight and then treated with EGF (50 ng/ml) for 30 minutes. Cell lysates were then analyzed for the phosphorylation of S208, S204, T179, and S213 by the phospho-peptide antibodies against each of these sites in immunoblots. Smad3 expression levels, ERK activities, and ERK levels were also analyzed in immunoblots.

(B) S208, S204, T179, and S213 in Smad3 can be phosphorylated by ERK2 *in vitro*. 26 ng of recombinant activated ERK2 was used to phosphorylate 0.3 μ M GST-Smad3 in 30 μ l. The reaction products were analyzed by immunoblotting with each of the pS208, pS204, pT179, and pS213 antibodies.

Figure legend continued on next page

EGF treatment (Fig 2.1A), we noticed that S208 showed the highest induction of phosphorylation by EGF in all the experiments. In addition, S208 is the only site in Smad3 that is recognized to be a MAP kinase phosphorylation site by the Scansite software program under high stringency condition (data not shown), which recognizes a wide variety of kinase phosphorylation sites and other signature motifs. The EGF dose curve and time course experiments also confirmed this notion. As shown in Fig 2.1C, S208 phosphorylation was significantly induced by EGF even at a dose of 1 ng/ml. In the EGF time course experiment in Fig 2.1D, S208 phosphorylation was markedly increased after treatment with EGF for only five minutes, whereas significant increase in phosphorylation on the S204 or T179 occurred only after treatment for fifteen minutes. Taken together, these observations indicate that S208 is the most sensitive site in Smad3 for MAP kinase phosphorylation.

2.4.3 MEK inhibitors suppress EGF-induced phosphorylation of S208, S204 and T179 in Smad3

To provide further evidence that MAP kinase phosphorylates S208, S204, and T179 in response to EGF, we asked whether EGF-induced phosphorylation at these sites can

Legend for Figure 2.1 continued

(C) EGF dose curve on S208, S204 and T179 phosphorylation in Smad3. Mv1Lu cells were treated with increasing concentrations of EGF as indicated for 30 minutes. Cell lysates were then analyzed using each of the antibodies against pS208, pS204, pT179, Smad3, pERK, and ERK.

(D) EGF time course of S208, S204 and T179 phosphorylation in Smad3. Mv1Lu cells were treated with 50 ng/ml EGF for the indicated period of time. Cell lysates were then analyzed with each of the antibodies against pS208, pS204, pT179, Smad3, pERK, and ERK.





Figure 2.2 MEK inhibitors PD98059 and U0126 inhibit ERK phosphorylation of S208, S204, and T179 in Smad3.

Mv1Lu cells were pretreated with 50 μ M PD98059 or 3 μ M U0126 for 1 hour prior to the addition of EGF. Cell lysates were then analyzed for S208, S204 and T179 phosphorylation by the corresponding phospho-peptide antibodies. Smad3 level, ERK activities, and ERK levels were also analyzed as controls. One representative experiment is shown. The averages of four experiments were plotted.

be inhibited by MEK1 inhibitors. Mv1Lu cells were pretreated with MEK1 inhibitor PD98059 at 50 μ M or U0126 at 3 μ M for one hour before addition of EGF. As shown in a representative experiment in Fig 2.2, PD 98059 and U0126 inhibited EGF-induced phosphorylation of S208, S204, and T179, confirming MAP kinase phosphorylation of these sites. The average of four experiments is plotted and presented in the lower panels of Fig 2.2.

2.4.4 Comparison of ERK2 phosphorylation of Smad3, MBP, and Elk1 in vitro

To determine whether Smad3 is a good substrate for ERK MAP kinase phosphorylation *in vitro*, we performed an *in vitro* kinase assay using recombinant ERK2 and GST-Smad3 as a substrate. For comparison, we also included myelin basic protein (MBP) and GST-Elk1 (307-428) as substrates in the same assay. Previous studies have shown that Elk1 is one of the best-characterized physiological substrates of ERK MAP kinase [178, 179] and MBP is also frequently used as a substrate to monitor ERK MAP kinase activity. Elk1 contains nine potential MAP kinase phosphorylation sites in its C-terminal domain [178, 179]. Phosphorylation of a major site, Ser 383, is critical for Elk1 transcriptional activity [178, 179]. Elk1 (371-428), the C-terminal domain of Elk-1, contains all the nine potential sites. Previous studies have also shown that Elk1 is a much better substrate than MBP with more than 5 fold difference in an *in vitro* kinase assay [180].



Figure 2.3 ERK2 titration for phosphorylation of Smad3, MBP, and Elk1 in vitro 1 μ M of GST-Smad3, MBP, or GST-Elk1 (307-428) were incubated with an increasing amount of recombinant activated ERK2 (3.75 ng, 7.5 ng, 15 ng, and 30 ng) in the presence of g-³²P-ATP at 30°C for 30 minutes. The upper panel is the autoradiogram of the kinase reaction. The lower panel is the same gel stained by Coomassie blue.

We performed an *in vitro* kinase assay using 1 μ M substrates and increasing amount of the recombinant ERK2 (3.75 ng, 7.5 ng, 15 ng, and 30 ng) at 30°C for 30 minutes. Fig 2.3 shows both the ³²P gel (upper panel) and the Coomassie blue-stained gel (lower panel). As seen from the Coomassie gel, Smad3 and MBP phosphorylation are in the linear range of all concentration of ERK2. The GST-Elk1 (307-428) phosphorylation led to significant shift in migration when 7.5 ng of ERK2 was used.

To determine the kinetic parameters of ERK2 for GST-Smad3, MBP, and GST-Elk1 (307-428), we performed substrate titration experiments using 3 ng ERK2 and increasing amount of substrates (0.1 μ M, 0.2 μ M, 0.4 μ M, 0.8 μ M, 1.6 μ M and 3.2 μ M) at 30°C for 20 minutes. Under this condition, phosphate incorporation was in a linear range over the time (data not shown). A representative ³²P gel is shown in Fig 2.4A. The phosphorylated bands were excised and counted for radioactivity, and the phosphate incorporation was plotted against substrate concentration and shown in Fig 2.4B. Phosphorylation of all three substrates by ERK2 follows the classic Michaelis-Menten kinetics, consistent with the previous conclusion that ERK2 phosphorylation of GST-Elk1 (307-428) and MBP obeys Michaelis-Menten kinetics [180]. The kinetic parameters of ERK2 for GST-Smad3, MBP, and GST-Elk1 (307-428) based on three experiments are shown in Table 2.1. The Km values for GST-Elk1 (307-428) and MBP are similar to those in a previous study [180]. The ratio of Vmax divided by Km is a good indicator for comparing substrates. This ratio for GST-Elk1 (307-428) is significantly higher than those of MBP and Smad3. The ratio of Vmax over Km for Smad3 is approximately four fold less than that of MBP.



Figure 2.4 Comparison of ERK2 phosphorylation of Smad3, MBP, and Elk1 in vitro (A) 3 ng ERK was used to phosphorylate increasing amount of GST-Smad3, MBP, or GST-Elk1 (307-428) (0.1 μ M, 0.2 μ M, 0.4 μ M, 0.8 μ M, 1.6 μ M and 3.2 μ M) in the presence of γ -³²P-ATP at 30°C for 20 minutes. A representative experiment is shown. (B) Phosphorylated GST-Smad3, MBP, and GST-ELK1 in (A) were excised and counted for radioactivity. The phosphate incorporation is plotted against substrate concentration.

Substrate	Km⁵	Vmax [°]	<u>Vmax</u> Km
	(μ M)	(pmole/min)	
GST-Elk1 (307-428)	1.3 ± 0.3	2.73 ± 0.60	1.52
Myelin basic protein	5.1 ± 1.0	1.95 ± 0.30	0.38
GST-Smad3	11.8 ±2.3	1.14 ± 0.19	0.10

Table 2.1Kinetic parameters of Smad3, MBP and Elk1 for ERK2

^a Average values from three experiments

^b Apparent value

° Velocity obtained from 3 ng recombinant ERK2

2.4.5 Analysis of the effect of MAP kinase phosphorylation on Smad3 subcellular localization

To determine whether MAP kinase phosphorylation affects Smad3 subcellular localization, Mv1Lu cells were serum-starved overnight and then treated with EGF. Cells were harvested and fractionated into cytoplasmic and nuclear fractions. As shown in Fig 2.5, EGF treatment resulted in increased levels of phosphorylated S208, S204, and T179 in both the cytoplasmic and nuclear fractions. The Smad3 protein levels in the cytoplasmic fraction and the nuclear fraction remained unchanged. ERK protein levels and pERK levels were also analyzed (Fig 2.5), consistent with previous observations that a small proportion of activated ERK moves into the nucleus [181]. The GAPDH (glyceraldehydes-3-phosphate dehydrogenase) served as a marker for cytoplasmic localization, whereas the PCNA (proliferating cell nuclear antigen) served as a marker for nuclear localization. The results in Fig 2.5 were from treatment of Mv1Lu cells with EGF for 15 minutes, which resulted in the highest phosphorylation of Smad3 based on the time course in Fig 2.1D. We also analyzed and found that treatment with EGF for 30 minutes or 1 hour did not change the amount of Smad3 in the cytoplasmic fraction and the nuclear fraction (data not shown). These observations suggest that MAP kinase phosphorylation does not affect Smad3 localization under our experimental conditions.

2.4.6 Mutation of the MAP kinase phosphorylation sites in Smad3 increases its ability to activate a Smad target gene

To determine the potential link between ERK MAP kinase phosphorylation and Smad3 activity, we mutated each of the three sites (T179, S204, and S208) to



Figure 2.5 EGF treatment does not affect Smad3 subcellular localization

Mv1Lu cells were serum-starved overnight and then treated with EGF (50 ng/ml) for 15 minutes. Cells were then harvested and fractionated into the cytoplasmic and nuclear fractions. Approximately same amount of total proteins were present in the cytoplasmic fraction versus in the nuclear fraction. Same amount of proteins from the cytoplasmic fraction and the nuclear fraction were loaded onto SDS gels and analyzed by immunoblot for pS208, pS204, pT179, Smad3, pERK, ERK levels. GAPDH and PCNA serve as cytoplasmic and nuclear markers, respectively.
nonphosphorylatable residues, either individually or in combination. We then analyzed their ability to activate the A3-luciferase reporter gene, a well-characterized Smad target gene. The A3 reporter gene contains in its promoter region DNA binding sites for Smads and for FAST-1, a winged-helix transcription factor. Smads together with FAST-1 activate the A3 reporter gene [157]. HepG2 cells were cotransfected with the A3-Luc reporter gene, FAST-1, and either wild type or a phosphorylation mutant form of Smad3. Cells were then analyzed for luciferase activity. As shown in Fig 2.6 (upper panel), these mutations increased the Smad3 capacity to stimulate the activity of the A3-Luc. The S208A is more active than S204A or T179V, consistent with S208 being the best MAP kinase site in Smad3. The triple mutant (T179V/S204A/S208A) and the double mutant (S204A/S208A) are more active than the single mutants in this assay. Examination of the protein levels indicated that these phosphorylation mutants were expressed at similar levels as the wild type Smad3 as shown in Fig 2.6 (lower panel).

To analyze whether the phosphorylation mutants have higher activities than the wild type Smad3 to stimulate an endogenous Smad target gene, we generated retroviruses to express the wild type and phosphorylation mutant forms of Smad3. The retroviruses were used to infect mouse C2C12 myoblasts, which contain very low levels of endogenous Smad3. Previous studies have shown that Smad3 plays an important role to activate the expression of ATF3 as part of the TGF-B/Smad antiproliferative responses [182]. As shown in Fig 2.7, wild type Smad3 activated ATF3 expression. Moreover, the phosphorylation mutant forms of Smad3 were more effective than the wild type Smad3 to activate ATF3 expression. Taken together, these observations suggest that ERK MAP kinase phosphorylation of Smad3 inhibits its activity.



 α -Smad3 blot

Figure 2.6 Mutation of the ERK phosphorylation sites in Smad3 increases its ability to activate a Smad reporter gene

HepG2 cells were cotransfected with the A3-Luciferase reporter gene, FAST1, and the vector control, wild type Smad3 or a phosphorylation mutant as indicated. Luciferase activity from the average of four independent transfection experiments is shown in the upper panel. One of the transfection experiments was also analyzed for Smad3 protein expression levels as shown in the lower panel. T-test was used to calculate the P values between wild type Smad3 versus each of the phosphorylation mutants. The results are listed here. T179V versus wild type Smad3: P < 0.001. S204A versus wild type Smad3: P < 0.001. S204A/S208A versus wild type Smad3: P < 0.001. S204A/S208A versus wild type Smad3: P < 0.001. T179V/S204A/S208A versus wild type Smad3: P < 0.001.



Figure 2.7 Smad3 phosphorylation mutants have increased abilities to upregulate ATF3 C2C12 cells were infected by retroviruses encoding wild type or a phosphorylation mutant Smad3, and then analyzed by immunoblot for ATF3 levels. The expression levels of Smad3 and the GAPDH were also analyzed by immunoblot as controls.

We have shown in this report that Smad3 is phosphorylated by ERK on S208, S204 and T179 in response to EGF treatment. S208 is the best ERK phosphorylation site in Smad3. Accordingly, S208 is the only site that is recognized as an ERK MAPK phosphorylation site by the Scansite program under high stringency condition. MEK1 inhibitors PD58059 and U0126 efficiently inhibited the phosphorylation, confirming that ERK MAP kinases phosphorylate these sites *in vivo*. We have also compared Smad3, Elk-1 and MBP phosphorylation by recombinant activated ERK2 in vitro, and determined their kinetic parameters. Mutation of the ERK phosphorylation sites in Smad3 increases its ability to activate a Smad target gene, suggesting that ERK phosphorylation of Smad3 inhibits its activity. We have recently shown that the Smad3 linker region contains a transcriptional activation domain [107]. Previous studies have reported that the interactions between MAP kinase superfamily and Smad could alter signaling and transcriptional responses in either positive or negative manner [46-49, 132-135, 138-141, 144-146, 148, 149, 172-174, 183-187]. Future studies are warranted to determine how phosphorylation by ERK and other kinases regulate Smad3 activity in a context-dependent manner, leading to distinct biological responses.

We have also examined whether EGF treatment affects Smad3 subcellular localization. The experiments were performed in Mv1Lu cells. Because we have not found the right conditions for the phospho-peptide antibodies against pS208, pS204, or pT179 in immunofluorescence studies, we carried out the analysis via cell fractionation. Mv1Lu cells were treated with EGF, and cell lysates were harvested and fractionated into cytoplasmic and nuclear fractions. We found that upon EGF treatment, phosphorylated S208, S204, and T179 were increased in both the cytoplasmic and nuclear fractions, consistent with the notion that activated ERK MAP kinases are present in both the cytoplasm and the nucleus. The Smad3 protein level in the cytoplasmic fraction and the nuclear fraction remained unchanged (Fig 2.5). These observations suggest that ERK phosphorylation does not affect Smad3 localization under our experimental conditions. A previous study showed that Ras-ERK pathway inhibits Smad3 nuclear accumulation [48]. Although we could not detect appreciable changes in Smad3 subcellular localization triggered by EGF treatment in Mv1Lu cells, it remains possible that ERK phosphorylation of Smad3 can affect its subcellular localization under other conditions. The role of ERK phosphorylation on Smads subcellular localization is a complex issue. Previous studies have shown that ERK phosphorylation of Smads can inhibit, promote, or have no effect on their subcellular localization, respectively [47-49, 133-135, 138, 188]. It is possible that ERK phosphorylation of a Smad can have a different effect on its subcellular localization in a cell context-dependent manner. Future studies are necessary to identify the underlying mechanisms.

EGF can also inhibit TGF-ß antiproliferative effects in a manner independent of Smad phosphorylation. For example, it has recently been shown that EGF inhibits TGF-ß upregulation of p15 in primary human ovarian cancer cells and this effect is independent of phosphorylation of Smad [188]. The mechanisms remain to be elucidated. It is speculated that TGIF, a Smad corepressor, may mediate this effect [188]. Previous studies have shown that ERK, activated by EGF treatment, phosphorylates TGIF and stabilize it, thus favoring the interaction between TGIF and Smad2 [189]. The

increased interaction of TGIF with Smad2 and Smad3 may contribute to the inhibition of the p15 promoter.

ERK and CDK may synergize for inhibition of Smad3 activity in a variety of biological processes as well as during tumorigenesis. We have previously shown that CDK phsophorylation inhibits Smad3 activity [50]. CDK and ERK have distinct as well as overlapping phosphorylation sites. CDK phosphorylates T8, T179 and S213 [50], whereas ERK phosphorylates S208, S204, and T179. Their phosphorylation converges on the T179. Cancer cells often contain higher levels of ERK and CDK activities [190, 191]. Full inhibition of Smad3 activity by ERK and CDK together may provide an important mechanism for tumorigenesis.

Chapter III

TGF-B-Inducible Phosphorylation of Smad3 Linker Sites

Smad proteins transduce the TGF- β signal at the cell surface into gene regulation in the nucleus. Upon TGF-ß treatment, the highly homologous Smad2 and Smad3 are phosphorylated by the TGF-B receptor at the SSXS motif in the C-terminal tail. Here we show that in addition to the C-tail, three (S/T)-P sites in the Smad3 linker region, S208, S204, and T179 are phosphorylated in response to TGF-B. The linker phosphorylation peaks at approximately 1 hour after TGF-B treatment, slightly behind the peak of the Ctail phosphorylation. We show that the C-tail phosphorylation by the TGF- β receptor is necessary for the TGF-B-induced linker phosphorylation. Although the TGF-B receptor is necessary for the linker phosphorylation, the receptor itself does not phosphorylate these sites. We further show that while ERK MAPK phosphorylates these three sites in response to EGF treatment, ERK is not responsible for TGF-B-dependent phosphorylation of these three sites. In addition, we show that JNK and p38 MAPK are unlikely to be the major kinases for these sites in the presence of TGF-B. GSK3 accounts for S204 phosphorylation and a small part of T179 phosphorylation, but not S208 phosphorylation, in response to TGF- β . Flavopiridol, a pan-CDK inhibitor, abolishes TGF-β-induced phosphorylation of T179 and S208, suggesting that one or more CDK family members phosphorylate T179 and S208 in response to TGF-β. Mutation of the linker phosphorylation sites to nonphosphorylatable residues increases the ability of Smad3 to activate a TGF-B/Smad-target gene, as well as the antiproliferative function of Smad3 protein. These observations suggest that TGF-Bdependent linker phosphorylation may inhibit Smad3 activity.

3.2 INTRODUCTION

TGF-ß and related factors regulate a wide variety of biological activities, such as cell proliferation, differentiation, migration, adhesion, apoptosis, angiogenesis, and immune function [155]. Accordingly, TGF-ß family members play an important role in early embryogenesis as well as in the homeostasis of adult tissues. Abnormalities in TGF-ß signaling lead to a number of human diseases, such as cancer and fibrosis [6, 192].

TGF-β signals through two types of serine-threonine kinase receptors [1, 4, 6, 156, 158, 192]. TGF-β binds directly to the type II receptor but not the type I receptor. TGF-β binding to the type II receptor leads to the recruitment of the type I receptor into the ligand-receptor complex. The type II receptor is constitutively active. It transphosphorylates and activates the type I receptor, which then plays a major role in specifying downstream signaling events [1, 4, 6, 156, 158, 192].

Smad proteins transduce the TGF-ß family signal at the cell surface into gene regulation in the nucleus [1, 4, 6, 156, 158, 192]. Smad2 and Smad3 are direct substrates of the TGF-ß type I receptor [14, 22, 23, 193], whereas Smad1, Smad5, and Smad8 are phosphorylated by BMP receptor kinase [12, 13]. These Smads are termed as receptor-regulated Smads (R-Smads). Upon TGF-ß treatment, Smad2 and Smad3 are phosphorylated by the TGF-ß type I receptor at the SSXS motif in their C-tails [22, 23], form complexes with Smad4, then together accumulate in the nucleus to regulate transcription of target genes [1, 4, 6, 156, 158, 192].

Smads contain conserved N-terminal and C-terminal domains, also designated as MH1 and MH2 domains, respectively. In the middle, there is a proline-rich linker region that is divergent in sequence and length [1, 4, 6, 156, 158, 192]. The R-Smads linker

regions contain demonstrated as well as suspected phosphorylation sites for prolinedirected kinases, such as cyclin-dependent kinases, ERK MAP kinases, c-Jun N-terminal kinases, and p38 MAP kinases, as well as for other kinases, such as Ca²⁺-calmodulindependent kinase II [46-50, 132-135, 138, 139, 143-145, 148, 149, 151, 154, 172-175, 184, 194-196]. The Smad3 linker region contains four proline-directed kinase phosphorylation sites: Thr 179 (T179), Ser 204 (S204), Ser 208 (S208), and Ser 213 (S213). Previous studies have shown that the Smad3 linker region can be phosphorylated by different kinases under different conditions. For example, CDK4 and CDK2 phosphorylate Smad3 at the Thr 8 (T8) in the N-terminal domain and T179 and S213 in the linker region [50]. CDK phosphorylation of Smad3 inhibits its transcriptional activity and antiproliferative function [50]. In response to EGF treatment, ERK MAPK phosphorylates S208, S204 and T179 in the Smad3 linker region and S208 is the best ERK site in Smad3 [50, 154]. JNK and p38 can phosphorylate certain sites in the Smad3 linker region in response to HGF or TGF-ß [144, 148, 149]. In addition, we have shown that the linker region contains a transcriptional activation domain [107]. An independent study has also shown that the linker region is necessary for Smad3 to activate transcription [106].

We show in this report that TGF- β treatment induces rapid phosphorylation of S208, S204, and T179 in the Smad3 linker region. Although phosphorylation of Smad3 at the C-tail SSXS motif by the TGF- β type I receptor is necessary for the linker phosphorylation, the receptor itself does not phosphorylate the linker region. We further show that ERK is not responsible for phosphorylation of the linker region in the presence of TGF- β . In addition, we provide evidence that JNK and p38 are unlikely to be

the major kinases for the TGF- β -induced linker phosphorylation. We show that the TGF- β -inducible phosphorylation of S204 is dependent on GSK3, whereas the TGF- β -induced phosphorylation of T179 and S208 requires one or more CDK family members. Mutation of the linker phosphorylation sites increases the ability of Smad3 to activate a TGF- β /Smad-responsive reporter gene. Smad3 mutants in which the linker phosphorylation sites were mutated have stronger antiproliferative activity than wild type Smad3 when they are introduced into Smad3^{-/-} MEF cells. Taken together, our observations suggest that TGF- β -induced phosphorylation of Smad3 linker sites may play an inhibitory role for Smad3 activity.

3.3 MATERIALS AND METHODS

3.3.1 Smad3 phosphopeptide antibodies and other antibodies

The Smad3 phosphopeptide antibodies against S208, S204, T179, and S213 were affinity purified against the phosphopeptide antigen, and cross-absorbed against the unphosphorylated peptide of the same sequence. The specificities of these phosphate-specific antibodies have previously been demonstrated by immunoblotting, immunoprecipitation, phosphatase treatment, and confirming that the recognized band is Smad3 by comparing wild type versus Smad3 deficient cells (Supplementary Fig. 4 in ref 50). In brief, each of the phosphopeptide antibodies recognizes only the wild type Smad3 but not the corresponding mutant Smad3 by immunoblotting; each of the phosphopeptide antibodies can recognize overexpressed wild type Smad3 but not the corresponding mutant form in an immunoprecipitation assay; treatment of the

phosphorylated Smad3 with the λ phosphatase leads to the disappearance of the phosphorylated band; the band recognized by each of the phosphopeptide antibodies is Smad3, as none of these antibodies can detect a band that comigrates with Smad3 using cell extracts from Smad3 knockout mouse embryonic fibroblasts (Supplementary Fig. 4 in ref 50). The Smad3 (C-tail) phosphopeptide antibody was from Biosource. The antibody against Smad3 was from Zymed laboratories. The ERK antibody was from Cell Signaling Solutions. The antibodies against phospho-ERK (pERK), JNK, and p38 were purchased from Santa Cruz Biotechnology, Inc. The antibodies against phospho-JNK (pJNK) and phospho-p38 (pp38) were from Cell Signaling Technology. The HA antibody was from Roche Applied Science.

3.3.2 Ligands and chemical inhibitors

For treatment of Mv1Lu or HaCaT cells with TGF- β , 300 pM or 200 pM of TGF- β was used unless otherwise indicated. The incubation time was usually for 1 hour or 45 minutes unless otherwise indicated. For treatment with EGF, Mv1Lu cells were treated with EGF (50 ng/ml) for 15 minutes, at which time the phosphorylation of the three sites were maximally induced (154). For treatment with MEK1 inhibitors PD98059 or U0126, Mv1Lu cells were pretreated with PD98059 (final concentration of 50 μ M) or U0126 (final concentration of 3 μ M) for one hour prior to the addition of EGF or TGF- β . For treatment with gSK3 inhibitor SB 203580, HaCaT cells were incubated with SB203580 (final concentration of 5 μ M) for one hour prior to the addition of TGF- β . For treatment with GSK3 inhibitor LiCl, Mv1Lu or HaCaT cells were incubated with 120 mM LiCl for 1 hour before adding TGF- β . For treatment with the CDK inhibitor flavopiridol, Mv1Lu

or HaCaT cells were incubated with 250 nM of flavopiridol for 8 hours before TGF- β treatment.

3.3.3 Immunoblotting

Immunoblotting was performed essentially as previously described [50, 154]. In brief, cells were lysed in the TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% NP-40) in the presence of protease and phosphatase inhibitors, and 30 μ g cell lysates were analyzed by immunoblotting. pS208, pS204, and pT179 antibodies were used at 0.15 μ g/ml. The concentrations of other antibodies were used according to manufacturers' instructions.

3.3.4 In vitro kinase assay

TBRII and TBRI-HA or TBRI (KR)-HA were cotransfected into 293 cells. 40 hours post transfection, cells were treated with TGF- β for 1 hour. Cells were harvested and lysed in the TNE buffer in the presence of protease and phosphatase inhibitors. The cell lysates were then immunoprecipitated with HA antibody. The immunoprecipitates were used in a nonradioactive kinase reaction containing 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.5 mM ATP, and 0.5 μ M GST-Smad3 in 30 μ l. The kinase reactions were carried out at 30°C for 30 min. The reactions were terminated by addition of SDS protein gel sample buffer. The reaction products were then analyzed by immunoblots.

3.3.5 Transfection and reporter gene assay

HepG2 cells were transfected and analyzed for luciferase activities as previously described [50]. In brief, HepG2 cells were seeded overnight in 60 mm dishes. Cells were transfected by DEAE-dextran (125 μ g/ml) for three hours. Cells were treated with TGFβ for 24 hours and luciferase activities were then analyzed. The luciferase activities were normalized by the cotransfected *Renilla* luciferase control pRL-TK (Promega). The results represent the mean and standard deviation of four independent transfection experiments.

3.3.6 ³H-thymidine incorporation assay

³H-thymidine incorporation assay was performed essentially as described previously (50). $2X10^5$ Smad3^{-/-} primary MEF and the wild-type littermate control MEF were seeded in six-well plates for 24 h, then treated for 24 h with or without 500 pM TGF- β . During the last 4 h, 5 µCi of 3H-thymidine was added to the culture, and radioactivity was assayed.

3.4 RESULTS

3.4.1 TGF-B induces rapid phosphorylation of the Smad3 linker sites

The Smad3 linker region contains four (S/T)-P phosphorylation sites: T179, S204, S208, and S213. We have generated phosphopeptide antibodies against each of the four phosphorylation sites in Smad3 [50]. The specificities of these phosphopeptide antibodies were previously demonstrated by immunoblotting, immunoprecipitation, phosphatase treatment, and confirming that the recognized band is Smad3 by comparing



Fig 3.1 TGF-β-inducible phosphorylation of three sites in Smad3 linker region

(A). Phosphorylation of three sites in Smad3 linker region is significantly induced by TGF- β . Mv1Lu, HaCaT and MEF cells were treated with 300 pM TGF- β for 1 hr. Phosphorylation of S207, S203 and T178 were detected by immunoblot using phosphospecific antibodies.

(B). Time course of TGF- β -inducible phosphorylation of Smad3. Mv1Lu cells were treated with 300 pM TGF- β for indicated period of time and phosphorylation of three linker sites as well as C-tail were analyzed by phosphospecific antibodies. Comparable amount of Smad3 was confirmed by Smad3 antibody.

(C). TGF- β dose curve of Smad3 linker sites phosphorylation. Mv1Lu cells were treated for 1 hr with indicated concentration of TGF- β . Phosphorylation of Smad3 linker sites was analyzed.

wild type versus Smad3 deficient cells (Supplementary Fig. 4 in ref 50). The availability of these phosphate-specific antibodies allowed us to analyze whether the Smad3 linker phosphorylation is regulated by TGF-ß treatment. Mv1Lu mink lung epithelial cells and HaCaT human keratinocytes are highly responsive to TGF-B and have been widely used in many studies on TGF-B. In addition, we subsequently will use mouse embryonic fibroblasts to study the antiproliferative function of wild type and mutant Smad3. Mv1Lu, HaCaT and MEF cells were treated with TGF-B for 1 hour. Cells were harvested and cell lysates were analyzed by immunoblotting with the Smad3 phosphopeptide antibodies. As shown in Fig 3.1A, endogenous Smad3 phosphorylation levels at three linker sites S208, S204 and T179 were induced by TGF-B treatment in Mv1Lu, HaCaT and MEF cells. We could not detect induction of endogenous S213 phosphorylation by TGF- β treatment in all three cell types (data not shown). The phosphopeptide antibodies against pS204 and pS208 recognize only Smad3, whereas the phosphopeptide antibody against pT179 also recognizes the analogous position in Smad2. As shown in Fig 3.1A, the phosphorylation of T220 in Smad2, which is in the analogous position as T179 in Smad3, is also significantly increased by TGF-B treatment. The subsequent studies are focused on the Smad3 linker phosphorylation.

3.4.2 The Smad3 C-tail phosphorylation is slightly ahead of the linker phosphorylation

We analyzed the time courses of TGF-B-induced Smad3 phosphorylation at the S208, S204, and T179 sites. For comparison, we also analyzed the Smad3 C-tail phosphorylation at the SSxS motif. As shown in Fig 3.1B, the time courses of S208, S204 and T179 phosphorylation are similar to that of the C-tail phosphorylation. The

increases of phosphorylation at the S208, S204 and T179 sites can be detected at \sim 10 minutes and peaks at \sim 45-60 minutes after TGF-ß treatment. The C-tail phosphorylation can be detected at \sim 5 minutes and peaks at \sim 30 minutes after TGF-ß treatment. Thus, the phosphorylation of C-tail slightly precedes the phosphorylation of the linker sites.

3.4.3 Low doses of TGF-B can induce the Smad3 linker phosphorylation

To determine whether the Smad3 linker phosphorylation can be induced by physiological concentrations of TGF- β , we performed TGF- β dose curve experiment. Mv1Lu cells were treated with different concentrations of TGF- β for 1 hour. The phosphorylation levels of S208, S204, and T179 were then analyzed by immunoblots. As shown in Fig 3.1C, as low as 10 pM of TGF- β was sufficient for almost maximal induction of phosphorylation at the S208, S204 and T179 sites. Thus, these sites are fully expected to be phosphorylated at physiological concentrations of TGF- β in living organisms.

3.4.4 The Smad3 C-tail phosphorylation is necessary for the TGF-β-induced linker phosphorylation

Since TGF-ß treatment leads to the induction of phosphorylation at the S208, S204 and T179 in the Smad3 linker region, we asked whether TßRI is required for the induction. As shown in Fig 3.2A, TGF-ß cannot induce Smad3 linker phosphorylation in L17 cells, a TßRI-deficient cell line derived from the parental Mv1Lu cell line. TGFß-dependent linker phosphorylation was restored when TßRI was introduced into L17 cells by transfection. This result indicates that the linker phosphorylation is mediated by



Fig 3.2 C-tail phosphorylation by T β RI is necessary for TGF- β -induced Smad3 linker sites phosphorylation.

(A). Requirement of T β RI for TGF- β -induced Smad3 linker phosphorylation. T β RIdeficient Mv1Lu- derivative L17 cells were transfected with T β RI. Following TGF- β treatment, phosphorylation of the linker sites was examined.

(**B**). C-tail phosphorylation of Smad3 is required for its TGF- β -induced linker phosphorylation. L17 cells were transfected with T β RI along with either wild type Smad3 or C-tail 3A mutant. Phosphorylation of the linker sites was analyzed as in (A).

(C). Mutation of the Smad3 linker sites does not affect its C-tail phosphorylation induced by TGF- β . L17 cells were transfected with T β RI along with either wild type or the linker sites mutant of Smad3. Phosphorylation of the C-tail was analyzed.

signaling events through the TGF-ß receptors. We next compared the TGF-ß-induced linker phosphorylation of wild type Smad3 with its C-tail phosphorylation mutant, Smad3 C-tail 3A. As shown in Fig 3.2B, phosphorylation of the linker sites was induced by TGF-ß in wild type Smad3, whereas the induction was abolished in C-tail 3A mutant. Thus, TßRI phosphorylation of the Smad3 C-tail is necessary for TGF-β-dependent phosphorylation of the linker sites. On the other hand, mutation of the three linker sites in Smad3 does not significantly affect the C-tail phosphorylation (Fig 3.2C), suggesting that the linker phosphorylation may not have a feedback role on the C-tail phosphorylation.

3.4.5 The TGF-B type I receptor is not the kinase for phosphorylation of the linker region

We next analyzed whether the TGF-ß type I receptor phosphorylates the linker sites. 293 cells were cotransfected by TßRII and HA tagged wild type TßRI or kinase-deficient TßRI (KR). Cells were then treated with TGF-ß, and cell lysates were immunoprecipitated by the HA antibody. The immunoprecipitates were subjected to a nonradioactive *in vitro* kinase assay using GST-Smad3 as a substrate. The reaction products were then analyzed by immunoblot with Smad3 phosphopeptide antibodies. In addition, the reaction products were analyzed by immunoblot with HA antibody to verify that the TßRI (wild type) and TßRI (KR) were present at very similar levels. As shown in Fig 3.3, the wild type TßRI markedly phosphorylated the C-tail of GST-Smad3. In contrast, the wild type TßRI was incapable of phosphorylating the linker sites of GST-Smad3. The background bands, which were detected after a long time exposure, were essentially at the same levels for TßRI (wild type) versus the kinase deficient TßRI

 Τβ RII Τβ RII TG F-β
 KR WT + +

 pSmad3 (C-tail)

 pS208

 pS204

 pS1179

 Tβ RI

 Tβ RI

 GST-Smad3

Fig 3.3 TβRI does not phosphorylate Smad3 linker sites.

HEK-293 cells were transfected with T β RII and HA-tagged wild type or a kinasedeficient mutant of T β RI (KR). Following TGF- β treatment, HA-T β RI was immunoprecipitated by HA antibody and subjected to an in vitro kinase assay using GST-Smad3 as a substrate. Phosphorylation of Smad3 C-tail and linker sites was analyzed with phosphospecific antibodies. (KR). Immunoblot analysis confirmed that TßRI (wild type) and TßRI (KR) were present at very similar levels (Fig 3.3). GST-Smad3 was also present at the same levels as confirmed by Coomassie blue staining (Fig 3.3). Thus, TßRI does not phosphorylate the Smad3 linker sites.

3.4.6 ERK MAP kinase is not responsible for phosphorylating the linker in response to TGF-β

We have previously shown that S208, S204, and T179 are phosphorylated by ERK MAP kinase in response to EGF treatment and S208 is the best ERK site in Smad3 [50, 154]. We therefore asked whether ERK is the kinase that phosphorylates the same three sites in the presence of TGF-B. We performed a TGF-B time course to monitor the changes in ERK activity, which was analyzed by a phosphopeptide antibody that detects only activated ERK. TGF-B induces a very rapid, transient, and modest activation of ERK in Mv1Lu cells. Upon TGF- β treatment, ERK activity is increased within 2 fold ~ 5 minutes after TGF-B treatment. The ERK activity then rapidly declines to basal level within minutes (data not shown). Our observations are consistent with a previous study on the time course of ERK activity in the presence of TGF-ß in Mv1Lu cells [197]. When TGF- β induces ~ maximal induction of phosphorylation at S208, S204 and T179 sites after incubation for 1 hour, ERK activity is essentially at basal level (Fig 3.4, compare lane 1 with lane 5 for the pERK panel). Moreover, pretreatment of cells with MEK1 inhibitors PD98059 or U0126 for 1 hour before addition of TGF-ß does not appreciably inhibit TGF- β -induced phosphorylation of the three sites (Fig 3.4).



Fig 3.4 ERK is not the kinase that phosphorylates the Smad3 linker region in response to TGF- β .

Mv1Lu cells were pretreated with 3 μ M U0126, 50 μ M PD98059 or DMSO (vehicle) for 1 hr before being treated with TGF- β for 1 hr or EGF for 15 min. Phosphorylation of the Smad3 linker sites was analyzed by phosphospecific antibodies. The level of phopho-ERK, an indicator of its activity, was analyzed by pERK antibody.

As positive controls, we have also prepared plates in parallel for treatment with EGF with or without pretreatment with a MEK1 inhibitor for 1 hour. As shown in Fig 3.4, EGF treatment activates ERK (compare lane 1 with lane 2 for the pERK panel), which is inhibited by pretreatment with MEK1 inhibitors. EGF treatment induces the phosphorylation at S208, S204, and T179, which is also inhibited by pretreatment with MEK1 inhibitors. Taken together, these results indicate that EGF treatment activates ERK, which phosphorylates S208, S204, and T179. The TGF-β-dependent phosphorylation of the same three sites, however, does not result from phosphorylation by ERK.

3.4.7 JNK is unlikely to be the major kinase phosphorylating the linker in response to TGF-β

We analyzed whether JNK phosphorylates the Smad3 linker in the presence of TGF- β . As shown in Fig 3.5A, TGF- β treatment transiently activated JNK, as analyzed by phospho-JNK (pJNK) levels. The JNK activity increases ~5 minutes after TGF- β addition and peaks at ~10 minutes. Its activity then rapidly declines, back to basal level after TGF- β treatment for ~20 minutes. On the contrary, the linker phosphorylation gradually increases until peaks at ~30-60 minutes after TGF- β treatment (Fig 3.5A). Based on the time course of JNK activity and the time courses of Smad3 phosphorylation at the three linker sites in the presence of TGF- β , JNK is unlikely to be the kinase that phosphorylates the sites. In addition, JNK is not activated by TGF- β in HaCaT cells (data not shown). We also examined whether SP600125, a chemical inhibitor for JNK, can inhibit the TGF- β -dependent linker phosphorylation. We observed



Fig 3.5 JNK is unlikely to be the kinase that phosphorylates the Smad3 linker sites in response to TGF- β .

(A). Mv1Lu cells were treated with TGF-β for indicated periods of time and phosphorylation of three linker sites was analyzed by phosphospecific antibodies. The level of phospho-JNK, an indicator of JNK activity, was analyzed by pJNK antibody.
(B). Induction of Smad3 linker phosphorylation in JNK1-/- or JNK2-/- MEFs and in wild type littermate control MEFs was analyzed by phosphospecific antibodies.

some inhibition of the Smad3 linker phosphorylation by the SP600125 inhibitor (data not shown). Because SP600125 is a relatively weak inhibitor for JNK and it also cross-inhibits 13 other kinases including CDK2 with similar or greater potency [198], the inhibition of Smad3 linker phosphorylation may not be due to the suppression of JNK activity. To further probe a possible role of JNK in phosphorylating Smad3 linker sites in response to TGF- β , we looked at the Smad3 linker phosphorylation in JNK1^{-/-} and JNK2^{-/-} MEFs. In JNK1 or JNK2 single knockout MEFs, the TGF- β induced phosphorylation on T179, S204, and S208 is not affected (Fig 3.5B). We did not examine JNK1-/-JNK2-/- MEFs because JNK1/JNK2 double knockout mice die early in development and the MEFs are very difficult to obtain and to grow. Taken together, our data does not support that JNK plays a role in TGF- β induced Smad3 phosphorylation on T179, S204, and S208.

3.4.8 p38 is unlikely to be the major kinase phosphorylating the linker in response to TGF-β

We examined the involvement of p38 in Smad3 linker phosphorylation. Four isoforms of p38: p38 α , p38 β , p38 γ , and p38 δ , are present in mammalian cells. As shown in Fig 3.6A and B, p38 is activated in response to TGF- β treatment, as analyzed by phospho-p38 (pp38) levels. We first tested the pan-p38 inhibitor BIRB in suppressing TGF- β induced Smad3 phosphorylation on T179, S204, and S208. BIRB pretreatment of cells significantly reduced p38 activity, but had little effect on the TGF- β inducible phosphorylation of T179, S204 and S208 (Fig 3.6A), suggesting that p38 is not a major kinase for the linker sites. SB203580, another chemical inhibitor for p38, also



Fig 3.6 p38 is unlikely to be the kinase that phosphorylates the Smad3 linker sites in response to TGF- β .

(A). HaCaT cells were pretreated for 2 hr with 10 mM BIRB or DMSO (vehicle) before TGF- β treatment for 45 min. Phosphorylation of three linker sites was analyzed by phosphospecific antibodies. The level of phospho-p38, an indicator of p38 activity, was analyzed by pp38 antibody.

(**B**). HaCaT cells were pretreated with 5 mM SB203580 or DMSO (vehicle) for 1 hr before treatment with TGF- β for 1 hr. Phosphorylation of three linker sites was analyzed by phosphospecific antibodies. The level of phospho-p38, an indicator of p38 activity, was analyzed by pp38 antibody.

(C). p38 α -/- MEF cells were treated with TGF- β for 45 min. Phosphorylation of the Smad3 linker sites was analyzed as above.

significantly inhibited p38 activity, whereas it had no effect on Smad3 linker phosphorylation (Fig 3.6B). p38 α accounts for 70-80% of the total activity of p38 (p38 α , p38 β , p38 γ , p38 δ). We looked at the TGF- β induced phosphorylation in p38 α ^{-/-} cells. We observed that TGF- β induced abundant phosphorylation on T179, S204, and S208 in p38 α ^{-/-} cells (Fig 3.6C). This suggests that p38 is not responsible for phosphorylating Smad3 linker sites in response to TGF- β .

3.4.9 MAP kinase phosphatase 1 (MKP1) inhibits ERK, JNK, and p38 but has little effect on the TGF-β-dependent linker phosphorylation

The results above argued against major involvement of ERK, JNK and p38 in the TGF-ß-inducible phosphorylation of the Smad3 linker sites. To further confirm this point, we transfected L17 cells with MKP1, a specific MAP kinase superfamily phosphatase that inactivates ERK, JNK and p38 by dephosphorylating them [199]. TGFß type I receptor was also cotransfected. Cells were treated with or without TGF-ß for 1 hour, and then analyzed for Smad3 linker phosphorylation and ERK, JNK, and p38 activities by pERK, pJNK, and pp38 levels, respectively. As shown in Fig 3.7, MKP1 effectively dephosphorylated and thus inactivated ERK, JNK, and p38. TGF-ß-dependent linker phosphorylation, however, was not significantly affected by the phosphatase. This result complements our conclusions above based on chemical inhibitors and time courses.

3.4.10 GSK3 is responsible for S204 phosphorylation in response to TGF- β

We tested the effect of lithium, a specific inhibitor for both GSK3 α and GSK3 β , on



Fig 3.7 MKP1 does not inhibit TGF-β-induced linker phosphorylation.

L17 cells were transfected with T β RI with or without MKP1, a MAP kinase phosphatase. Cells were treated with TGF- β for 1 hr. Phosphorylation of the Smad3 linker region was analyzed with phosphospecific antibodies. pERK, pJNK, and pp38 level was analyzed by the corresponding antibodies.

TGF- β induced Smad3 linker phosphorylation. Pretreatment of cells by lithium chloride (LiCl) completely abolished TGF- β induced S204 phosphorylation, slightly reduced T179 phosphorylation, and increased S208 phosphorylation (Fig 3.8A). This indicates that GSK3 is responsible for TGF- β induced S204 phosphorylation and a small proportion of T179 phosphorylation. GSK3 is completely dispensable for TGF-β induced S208 phosphorylation. We also examined the TGF-β-inducible Smad3 linker phosphorylation in GSK3^{B^{-/-}} MEFs. As expected, knocking out GSK3^β reduced S204 phosphorylation by ~80%, but only slightly reduced T179 phosphorylation, and even increased TGF-β-induced S208 phosphorylation (Fig 3.8B). The fact that knocking out GSK3 β did not completely abolish S204 phosphorylation suggests that both GSK3 α and GSK3ß contribute to S204 phosphorylation in response to TGF-ß treatment. In some cases, phosphorylation of a specific Ser/Thr residue by GSK3 requires the prephosphorylation on another Ser/Thr residue at n+4 position. Interestingly, the positioning of S208 and S204 exactly fits the n+4 rule, i.e. S208 is four amino acid downstream of S204. We asked whether S208 phosphorylation is required for S204 phosphorylation. In the S208A mutant in which the S208 phosphorylation is abolished, the S204 site is no longer phosphorylated (Fig 3.8C). This supports the notion that S208 is a priming site for S204 phosphorylation by GSK3.

3.4.11 Kinase(s) phosphorylating T179 and S208 in response to TGF- β likely belong to the CDK family

We have previously demonstrated that G1 CDKs (CDK2, 4, 6) phosphorylate Smad3 on Thr 8, Thr 179, and Ser 213 in the absence of TGF- β . It is possible that in the



Fig 3.8 GSK3 is the kinase for TGF-β-inducible phosphorylation of S204.

(A). LiCl selectively inhibits S204 phosphorylation. Mv1Lu cells were pretreated with 120 mM NaCl (control) or LiCl for 1 hr before addition of TGF- β . The Smad3

linker phosphorylation was analyzed by phosphospecific antibodies.

(B). TGF- β -induced phosphorylation of S204 is selectively attenuated in GSK3 β -/-MEF. Wild type littermate control MEF and GSK3 β -/-MEF are treated with TGF- β for 45 min. Phosphorylation levels of the three linker sites were examined.

(C). S204 phosphorylation is dependent on S208 phosphorylation. L17 cells were transfected with wild type Smad3 or S208A mutant, together with T β RI. Following TGF- β treatment, cell lysates were analyzed for S204 phosphorylation by phosphospecific antibody.

presence of TGF- β some CDKs or related family members may also phosphorylate the TGF- β inducible sites Thr 179 or Ser 208. The CDK family contains not only the cell cycle CDKs (CDK1, 2, 4, 6) [212-216] but also other CDKs functioning in transcriptional regulation, neuronal activity, splicing, and apoptosis (e.g. CDK7, 8, 9; CDK5; CDK10, 11) [217-229]. We used a pan-CDK inhibitor flavopiridol to investigate whether TGF- β induced phosphorylation of T179 and S208 depends on members of the CDK family. As shown in Fig 3.9, pretreatment of cells with flavopiridol before adding TGF- β completely abolished TGF- β induced phosphorylation by flavopiridol is not surprising, since flavopiridol abolished S208 phosphorylation, which is required for S204 phosphorylation. In addition, flavopiridol also inhibits GSK3 at higher concentrations. What is striking is that both T179 and S208 phosphorylation is abolished by flavopiridol. Since flavopiridol mainly inhibit kinases of the CDK family, the kinases that phosphorylate T179 and S208 very likely belong to the CDK family.

3.4.12 Mutation of the linker phosphorylation sites increases the ability of Smad3 to activate a TGF-B/Smad reporter gene and the antiproliferative activity of Smad3

To determine whether the linker phosphorylation affects the ability of Smad3 to regulate a TGF-B/Smad responsive gene, we mutated the phosphorylation sites individually or in combination, and then analyzed their ability to activate the A3-Luc reporter gene, which represents one of the best characterized TGF-B/Smad target gene. The A3 reporter gene contains DNA binding sites for Smads and for FAST-1, a winged-helix transcription factor. Smads and FAST-1 together activate the A3 reporter gene



Fig 3.9 Flavopiridol, a pan-CDK and GSK3 inhibitor, inhibits TGF- β induced linker phosphorylation, but not C-tail phosphorylation.

Mv1Lu cells were treated with or without 250 nM flavopiridol for 8 hr. Cells were then treated with TGF- β for 45 min and phosphorylation of Smad3 linker sites was analyzed.

[200, 201]. As shown in Fig 3.10A, the linker phosphorylation mutants have increased capacity to activate the A3-Luc reporter gene. Immunoblot analysis indicated that the mutants and the wild type Smad3 were expressed at similar levels.

To determine the effect of Smad3 linker phosphorylation on its antiproliferative function, we introduced wild type or mutant Smad3 into Smad3^{-/-} MEFs. Cell growth inhibition was monitored by ³H-thymidine incorporation assay. As shown in Fig 3.10B, when the T179, S204, or S208 were mutated singly or in combination, the resulting mutants inhibit the proliferation of MEFs more strongly than wild type Smad3. Taken together, these observations suggest that TGF-B-mediated phosphorylation of the linker region inhibits Smad3 activity.

3.5 DISCUSSION

Smads transduce the TGF-ß signal at the cell surface into gene regulation in the nucleus [1, 4, 6, 156, 158, 192]. We have shown in this report that in addition to the C-tail phosphorylation, TGF-ß induces Smad3 phosphorylation at the linker region. Importantly, the C-tail phosphorylation by the receptor is necessary for the linker phosphorylation in the presence of TGF-ß. The kinetics of the linker phosphorylation slightly ahead of the linker phosphorylation. Low doses of TGF-ß are sufficient to induce the linker phosphorylation, strongly suggesting that the phosphorylation occurs in living organisms at physiological concentrations of TGF-ß. Mutation of the phosphorylation sites increases the ability of Smad3 to activate a TGF-ß/Smad target gene in transient transfection assays, suggesting that TGF-β-induced linker phosphorylation may inhibit



Fig 3.10 Mutation of the Smad3 linker phosphorylation sites increases its activity.

(A). Mutation of the Smad3 linker sites increases its ability to activate a Smad target gene in transient transfection. HepG2 cells were co-transfected with the A3-Luciferase reporter gene, FAST-1, and the vector control, wild type Smad3 or a phosphorylation mutant as indicated. Cells were then treated with TGF- β . Luciferase activity from the average of four independent experiments is shown in the upper panel. Smad3 expression levels were examined in each experiment and a representative Smad3 immunoblot is shown in the lower panel.

(B). Mutation of the Smad3 linker sites increases its growth inhibitory activity. Smad3 or its phosphorylation mutants were introduced into Smad3^{-/-} MEF by retroviral infection. Cells were treated with or without TGF- β for 24 hr. ³H-thymidine was added for the last 4 hr of the incubation. 3H-thymidine incorporated into the cells was measured by scintillation counting.

Smad3 transcriptional activity.

We identified TGF- β -inducible phosphorylation sites in the Smad3 linker region at S208, S204, and T179. The S208 and S204 phosphopeptide antibodies do not recognize the analogous positions in Smad2. The T179 phosphopeptide antibody also recognizes the analogous position (T220) in Smad2, and revealed that Smad2 is phosphorylated at T220 in response to TGF- β . Carefully reviewing published work revealed that a previous study had shown that in response to TGF- β treatment, a phosphopeptide containing the linker region of Smad2 is phosphorylated as analyzed by 2-dimensional phosphopeptide mapping [22]. Smad3 and Smad2 are highly homologous. They have overlapping as well as distinct functions [22, 157]. It is not clear whether the TGF- β -induced phosphorylation of the Smad2 linker region restricts only to those sites that are analogous to the Smad3 sites. Future studies are necessary to identify all the TGF- β -induced phosphorylation sites in Smad2.

We have shown that the TGF-ß receptor is necessary for the TGF-ß-induced linker phosphorylation, but the TGF-ß receptor itself does not phosphorylate the linker sites, in contrast to the robust phosphorylation of the C-tail (Fig 3.3). Since the phosphorylated serines at S208 and S204 and phosphorylated threonine at T179 are followed by prolines, it is most likely that proline-directed kinases phosphorylate these sites. However, we cannot exclude the possibility at the present time that a non-proline directed kinase may phosphorylate a certain site.

The proline-directed kinases include 61 members that belong to the CDK, MAPK, GSK, and CDK-like families [202]. The MAPK family includes various isoforms of ERK, JNK, and p38. We analyzed whether ERK is the kinase that phosphorylates the

linker sites in the presence of TGF-B. Previous studies have shown that TGF-B can significantly activates ERK in a cell type-dependent manner. For example, TGF-B stimulates articular chondrocytes proliferation [203]. In response to TGF-B, ERK is rapidly and markedly activated in articular chondrocytes [203]. Its activity peaks 5 minutes after TGF-ß treatment and then declines to basal level in 4 hours. In Mv1Lu cells, TGF-ß potently inhibits their proliferation. TGF-ß treatment causes a transient and slight activation of ERK in Mv1Lu cells: the activation is within 2 fold; ERK activity peaks \sim 5 minutes after TGF- β treatment and then rapidly declines to basal level within minutes. This is consistent with previously reported time course and extent of ERK activation by TGF-B in Mv1Lu cells [197]. Pretreatment of cells with a MEK1 inhibitor have little effect on Smad3 linker phosphorylation in the presence of TGF-β (Fig 3.4). In addition, MKP1, which effectively dephosphorylates and inactivates ERK and other members of the MAPK family, cannot appreciably inhibit TGF-B-induced phosphorylation (Fig 3.7). Thus, although ERK phosphorylates S208, S204 and T179 in response to EGF treatment [50, 154], ERK is not responsible for TGF-B-dependent phosphorylation of the same three sites.

We also analyzed the involvement of JNK and p38 in TGF- β -induced phosphorylation. Although JNK is activated in response to TGF- β in Mv1Lu cells, its activation is transient. JNK activity already declines to the basal level while the linker phosphorylation level is still increasing (Fig 3.5). The time course experiments suggest that JNK is unlikely to play a major role in phosphorylation of the Smad3 linker sites. Moreover, JNK is not activated by TGF- β in HaCaT cells. Although the JNK inhibitor SP600125 can inhibit the linker sites phosphorylation to certain degree (data not shown),
SP600125 is a relatively weak inhibitor of JNK isoforms and it cross-inhibits 13 out of 28 kinases analyzed with similar or even greater potency than inhibition of JNK [198]. For example, CK18, DYRK1A, CDK2, SGK, S6K1, and AMPK are all inhibited to a grater extent than JNK by SP600125 [198]. Thus, the effect of SP600125 may not be due to specific inhibition of JNK. Moreover, in JNK1 or JNK2 single knock-out cells, the TGF- β induced Smad3 linker phosphorylation is not affected. The above results suggest that JNK is unlikely to phosphorylate T179, S204, or S208 in response to TGF-β. p38 is activated in response to TGF-B treatment (1, and Fig 3.6A, B). When p38 activity is significantly inhibited by the chemical inhibitor BIRB or SB203580, the TGF-B-induced linker phosphorylation is unaffected (Fig 3.6A, B). Since the p38 inhibitor SB230580 also cross-inhibits TGF- β type I receptor when present at higher than 10 μ M, this prevents us from using high doses of SB230580. Consistent with kinase inhibition data, the TGF- β induced phosphorylation of Smad3 is not affected in p38 $\alpha^{-/-}$ MEFs (Fig. 3.6C). Importantly, MKP1, which dramatically reduces the levels of activated JNK and p38 as well as ERK, does not have a significant effect on the TGF-β-induced linker phosphorylation (Fig 3.7). This observation provides persuasive evidence that JNK and p38 are not the major kinases for phosphorylation of the linker sites in the presence of TGF-B. It is important to emphasize that we do not exclude the possibility that under certain circumstances, JNK and p38 can play a role in TGF-B-induced Smad3 linker phosphorylation in a cell context-dependent manner. Our studies do indicate that JNK and p38 are usually not the major kinases for phosphorylation of the Smad3 linker sites in the presence of TGF- β .

We have previously shown that T179 in Smad3 is a phosphorylation site for cyclin D-CDK4 and cyclin E-CDK2 [50]. CDK6 is highly homologous to CDK4, and it is fully expected that cyclinD-CDK6 also phosphorylate T179. Thus far, we cannot conclude whether CDKs are the kinases that phosphorylate T179 in the presence of TGF- β . Mouse knockout studies of all three D-types of cyclin, both CDK4 and CDK6, both E-types of cyclin, and CDK2 revealed that we know little of the compensatory mechanisms that may operate in the absence of a cyclin or a CDK [204-211]. To unequivocally answer the question whether CDKs are the kinases that phosphorylate T179 in the presence of TGF- β , it is necessary to delete or dramatically reduce the levels of all these three CDKs. CDK2 and CDK4 double knock-out MEF cells can be obtained [230], we will knock down CDK6 in these cells and examine whether the TGF- β inducible phosphorylation of Thr 179 and Ser 208 is affected.

It is important to emphasize that although different kinases can phosphorylate the same sites, the requirement for phosphorylation by different kinases as well as the consequence of the phosphorylation can be different. For example, CDK4 and CDK2 phosphorylate Smad3 in the absence of TGF-ß when Smad3 is likely in a closed conformation with the N-terminal domain and the C-terminal domain interacting with each other [64]. It is possible that such closed conformation facilitates CDK to dock on Smad3 directly or indirectly and to phosphorylate a particular site. In the presence of TGF-ß, however, the C-tail is phosphorylated. The C-tail phosphorylation leads to the disruption of the interaction between the N-terminal domain and C-terminal domain, resulting in Smad3 to assume an open conformation. When Smad3 is in an open conformation, certain kinases, such as CDK, may not be able to appropriately dock on

Smad3, thus unable to phosphorylate some or all of the sites that can be phosphorylated in the absence of TGF- β . Thus, although the same site can be phosphorylated by different kinases in the basal state or in the presence of TGF- β , the phosphorylation by a kinase at basal state versus a kinase in the presence of TGF- β may play differential roles in regulation of Smad3 activity.

We have used a wide variety of chemical inhibitors to probe the potential kinases. Lithium, a potent and specific GSK3 inhibitor, abolished TGF- β inducible phosphorylation of S204 (Fig 3.8). S204 phosphorylation reduced by 80% in GSK3 β ^{-/-} MEFs, suggesting that GSK3 α and GSK3 β both contribute to TGF- β induced S204 phosphorylation. In addition, phosphorylation of S208 is required for S204 phosphorylation.

The phosphorylation of T179 and S208 can be abolished by flalvopiridol, a pan-CDK inhibitor (Fig 3.9). There are at least 11 members in the CDK family. Since flavopiridol strongly inhibits activity of all CDK family members, it is not clear which CDK(s) phosphorylate T179 and S208 in response to TGF-β. Members of the CDK family have very diverse cellular functions. CDK1 controls mitotic phase of the cell cycle [212-214]. CDK2, CDK4 and CDK6 are G1 CDKs that control cell cycle progression from G1 to S phase [215, 216]. CDK5 regulates several neuronal activities. CDK7, 8, and 9 are mainly involved in transcription regulation [217-222]. All three of them can phosphorylate the C-terminal domain (CTD) of RNA polymerase II large subunit [217]. Cdk7/cyclin H complex is a component of TFIIH [217-218]. In addition, the CDK7/cyclinH/MAT1 complex is essential for the activating phosphorylation of cell cycle CDKs (CDK1, 2, 4, 6). The CDK8/cyclin C complex is present as subunits of the mediator complex for transcriptional activation [217-220]. CDK9/cyclinT/K is the positive transcription elongation factor p-TEFb. By phosphorylating Ser-2 of CTD it is essential for the transition of RNA polymerase II from initiation phase to elongation phase [217-219, 221-222]. CDK10 functions in the G2/M phase of the cell cycle [223]. CDK11 associates with cyclin L. CDK11 has three forms (p46, p58, and p110) that are generated from two essentially same genes. Different forms of CDK11 can function in transcription, splicing, G2/M phase, and in apoptosis [218-219, 224-229]. Further study is needed to determine which CDK(s) are responsible for S208 and T179 phosphorylation.

We have analyzed the effect of TGF-ß-inducible linker phosphorylation on Smaddependent transcription. We have shown that mutation of the TGF-ß-inducible linker phosphorylation sites increases the ability of Smad3 to activate the A3-Luc reporter gene (Fig 3.10A), suggesting that phosphorylation inhibits Smad activity. When the wild type or linker phosphorylation sites mutant Smad3 were introduced to Smad3^{-/-} MEFs, mutant Smad3 had significantly higher activity to repress the proliferation of MEFs (Fig 3.10B).

In conclusion, we have identified three sites in the Smad3 linker region that are rapidly phosphorylated by TGF- β treatment. The C-tail phosphorylation by the receptor is necessary for TGF- β -induced phosphorylation of the linker region. Our observations reveal that the main scheme of the TGF- β signal transduction pathways are more complex than previously envisioned. Our findings provide an important foundation for further identification of novel key components in the TGF- β signal transduction pathways.

Chapter IV: The Smad3 Linker Region Contains a

Transcriptional Activation Domain

4.1 ABSTRACT

TGF-B/Smads regulate a wide variety of biological responses through transcriptional regulation of target genes. Smad3 plays a key role in TGF-B/Smads-mediated transcriptional responses. Here we show that the proline-rich linker region of Smad3 contains a transcriptional activation domain. When the linker region is fused to a heterologous DNA binding domain, it activates transcription. We show that the linker region physically interacts with p300. The adenovirus E1a protein, which binds to p300, inhibits the transcriptional activity of the linker region, and overexpression of p300 can rescue the linker-mediated transcriptional activation. In contrast, an adenovirus E1a mutant, which cannot bind to p300, does not inhibit the linker-mediated transcription. The native Smad3 protein lacking the linker region is unable to mediate TGF-B transcriptional activation responses, although it can be phosphorylated by the TGF-B receptor at the C-tail and has a significantly increased ability to form a heteromeric complex with Smad4. We further show that the linker region and the C-terminal domain of Smad3 synergize for transcriptional activation in the presence of TGF-B. Thus, our findings uncover an important function of the Smad3 linker region in Smads-mediated transcriptional control.

4.2 INTRODUCTION

TGF-β regulates a wide variety of biological activities, such as cell proliferation, differentiation, adhesion, motility, and apoptosis [155]. It signals through two types of transmembrane serine/threonine kinase receptors [1, 4, 156-158]. The type II receptor is constitutively active. Upon TGF-β binding, the type II receptor phosphorylates the type I

receptor, which then plays a major role in specifying downstream events [1, 4, 156-158]. Smad proteins are direct substrates of the TGF-ß family type I receptor and can transduce the signals at the cell surface into transcriptional responses in the nucleus, leading to various biological effects [1, 4, 156-160].

Based on structural and functional characteristics, the Smad family can be divided into distinct groups [1, 4, 156-160]. One group includes those pathway-specific Smads, termed R-Smads, which are phosphorylated by receptor kinases. For example, Smad2 and Smad3 are phosphorylated by the TGF- β receptor kinase. The second group includes the common Smads, designated Co-Smads, which participate in various TGF- β family members signaling pathways. The only known member of this group in vertebrates is Smad4. In response to TGF- β treatment, Smad2 and Smad3 are phosphorylated by the activated TGF- β receptor at the SSXS motif in the C-tail, form complexes with Smad4 and together accumulate in the nucleus to regulate transcription of target genes [1, 4, 156-160]. Smads can activate transcription by recruitment of coactivators, such as p300/CBP, P/CAF, ARC105, MSG1, and SMIF [35, 37-41, 105, 108, 159-164]. On the other hand, Smads can also inhibit transcription by recruitment of corepressors [160, 165, 166].

The R-Smads and Co-Smads contain conserved N-terminal and C-terminal domains linked by a proline-rich region, termed linker region, which is divergent in sequence and in length [1, 4, 156-160]. The N-terminal domains of Smad3 and Smad4 possess intrinsic DNA binding activities [4, 157, 160]. Although Smad2 is highly homologous to Smad3 [4, 157], Smad2 cannot bind to DNA due to the interference of extra 30 amino acids encoded by a separate exon present immediately before the DNA-binding hairpin [4, 157]. While Smad3 and Smad4 possess DNA binding activities, their affinities for most natural TGF-B-responsive promoters are not high enough to allow them to bind on their own. Instead, Smads are usually recruited to target promoters through interaction with DNA binding factors [4, 156, 157, 159, 160]. The C-terminal domains of Smads are responsible for homo-oligomerizations for R-Smads and Co-Smads and for hetero-oligomerizations between a R-Smad and a Co-Smad [1, 4, 156-160]. Moreover, the C-terminal domains of Smad2 and Smad3 have been shown to participate in transcriptional activation through recruitment of coactivators, such as p300/CBP [35, 38-41, 108, 161, 162]. Smad4 also plays an essential role in Smads-mediated transcriptional regulation [1, 4, 156-160]. This is due to, at least in part, the presence of a Smad activation domain (SAD) in the linker region [37]. The SAD physically interacts with coactivators, such as p300/CBP [37, 105, 163, 164]. The function of the linker regions of Smad2 and Smad3 had not been well explored in transcriptional control.

In this report, we show that the Smad3 linker region contains a transcriptional activation domain. It physically and functionally interacts with p300. Moreover, we show that the Smad3 linker region cooperates with the C-terminal domain for TGF-ß-inducible transcriptional activation.

4.3 MATERIALS AND METHODS

4.3.1 Plasmid constructions

GAL4-Smad3 (Linker), GAL4-Smad3 (C), GAL4-Smad3 (LC), and GAL4-Smad3 (Full Length) were constructed by inserting DNA fragments encoding Smad3 amino acids 142-230, 231-424, 142-424, and 1-424, respectively, into the pSG424 vector [167], which encodes the GAL4 DNA binding domain. Myc-tagged Smad3 (Linker) and Smad3 (Full Length) were constructed in the CS3⁺-6Myc vector [41]. Smad3 (NC) was constructed by inserting DNA fragments encoding Smad3 amino acids 1-141 and amino acids 231-424 into the CS2 vector. GAL4-SAD (37), E1a, E1a (Δ 2-36), and p300-HA [40] were described previously.

4.3.2 Cell culture and antibodies

Mink lung epithelial Mv1Lu/L17 cells were maintained in MEM containing nonessential amino acids (NEAA), 10% dialyzed fetal bovine serum (FBS) and histidinol. Human hepatoma HepG2 cells were cultured in MEM-NEAA-10% FBS with 1 mM sodium pyruvate. Human HaCaT keratinocytes were maintained in MEM-NEAA-10% FBS. COS and 293 cells were cultured in DME-10% FBS. All cell culture media contained 2 mM glutamine, 100 IU/ml penicillin and 100 μ g/ml streptomycin. All cells were grown at 37⁰C in chambers supplied with 5% CO₂. Antibodies against the GAL4 DNA binding domain (Upstate Signaling Solutions), the HA epitope (Roche Diagnostics), the Myc epitope (Sigma), the Smad3 linker region (Zymed Laboratories), the Smad3 C-terminal domain (Santa Cruz Biotechnolgy, Inc), and the Smad3 C-tail phosphorylation sites (Biosource) were used.

4.3.3 Transfection and reporter Gene Assay

Mv1Lu/L17, HepG2, HaCaT, and COS cells in 60 mm dishes were transfected by DEAE-dextran. 293 cells were transfected by liopfectamine plus reagent. Cells were treated with or without 500 pM TGF-β for 18-24 hours and then analyzed for luciferase activity as previously described [168]. Luciferase activities were normalized by the cotransfected *Renilla* luciferase control driven by pRL-TK (Promega). Results represent the mean and standard deviation of at least three independent transfection experiments.

4.3.4 Immunoprecipitation and immunoblot

Immunoprecipitation and immunoblot were performed essentially as previously described [169]. In brief, to detect an interaction between Smad3 linker region and p300, 293 cells were transfected by lipofectamine plus reagent. Cells were lysed in the TNM buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 3 mM MgCl₂, 0.5% NP-40) in the presence of protease and phosphatase inhibitors, and immunoprecipitated by appropriate antibodies. The immunoprecipitates were washed five times each with 1 ml buffer for 10 min and then loaded on a gel and immunoblotted by appropriate antibodies. To detect GAL4 fusion protein expression levels, immunoblot with an antibody against the GAL4 DNA binding domain was performed as described previously [168].

4.4 RESULTS

4.4.1 The Smad3 linker region has an intrinsic transcriptional activity

To determine whether the Smad3 linker region contains a transcriptional activation domain, we fused the linker region to the GAL4 DNA binding domain. As shown in Fig 4.1A, the GAL4-Smad3 (Linker) activates transcription in a dose-dependent manner.



Figure 4.1 The Smad3 linker region contains a constitutive transcriptional activation domain.

(A). GAL4 (DBD) (0.8 μ g DNA) or GAL4-Smad3 (Linker) (0.05 μ g, 0.1 μ g, 0.2 μ g, 0.4 μ g, or 0.8 μ g DNA) were cotransfected with a GAL4 reporter gene (1.6 μ g) into Mv1Lu/L17 cells in 60 mm dishes, and analyzed for luciferase activity. GAL4 (DBD) refers to GAL4 (DNA binding domain).

(**B**). The transcriptional activity of Smad3 linker region is comparable to that of SAD. GAL4 (DBD), GAL4-Smad3 (Linker), or GAL4-SAD were cotransfected with a GAL4 reporter gene into COS cells. Cell lysates were analyzed for luciferase activity. Protein expression levels were examined by immunoblot with an antibody against the GAL4 DNA binding domain.

(C). The transcriptional activity of the Smad3 linker region is constitutive. GAL4 (DBD), GAL4-Smad3 (Linker), or GAL4-Smad3 (Full Length) were cotransfected with a GAL4 reporter gene and analyzed for luciferase activity.

GAL4-Smad3 (Linker) has a transcriptional activity in various cell lines we examined, including Mv1Lu/L17, HepG2, HaCaT, COS and 293 cells (Fig 4.1A and data not shown). When compared with the GAL4-SAD, we found that the Smad3 linker region has a transcriptional activity comparable with that of SAD (Fig 4.1B). GAL4-Smad3 (Linker) and GAL4-SAD were expressed at comparable levels (Fig 4.1B). Similar to the SAD [37], the transcriptional activity of Smad3 linker region is also constitutive; TGF- β treatment has little effect on its activity (Fig 4.1C). This is contrast to the transcriptional activity of GAL4-Smad3 (Full Length), which is significantly induced by TGF- β (Fig 4.1C). Intramolecular interactions between the N-terminal and C-terminal domains [26] may inhibit the transcriptional activity of GAL4-Smad3 (Full Length) at basal state.

4.4.2 The Smad3 linker region functionally interacts with p300

To determine whether the Smad3 linker region functionally interacts with p300, we analyzed the effect of the adenovirus E1a protein, which interacts with p300 and inhibits p300-mediated transcriptional activation [170]. As shown in Fig 4.2A, wild type E1a potently inhibited the transcriptional activity of the GAL4-Smad3 (Linker). In contrast, E1a (Δ 2-36), which cannot bind to p300 (ref 170), has little effect. To provide further evidence for the functional requirement of p300 in Smad3 linker-mediated transcription, we asked whether overexpression of p300 can rescue the inhibitory effect of E1a. As shown in Fig 4.2B, overexpression of p300 rescued the inhibition in a dose-dependent manner to a large extent. Overexpression of p300 at higher doses did not result in complete rescue (data not shown). Similarly, previous studies found that overexpression



Figure 4.2 The linker region of Smad3 functionally interacts with p300.

(A). E1a inhibits the Smad3 linker-mediated transcriptional activation. Mv1Lu/L17 cells were cotransfected with the GAL4 reporter gene, GAL4 (DBD), GAL4-Smad3 (Linker), in the absence or presence of wild type E1a or E1a (Δ 2-36) as indicated, and analyzed for luciferase activity.

(B). p300 can rescue the inhibitory effect of E1a to a large extent. 293 cells were cotransfected with the GAL4 reporter gene, GAL4-Smad3 (Linker), E1a, and different amount of p300 as indicated, and analyzed for luciferase activity.

of p300 partially restored E1a-mediated repression of GAL4-SAD transcriptional activity [37]. Although it is not clear why it is difficult to achieve complete rescue, the results in Fig 4.2 suggest that p300 participates in Smad3 linker-mediated transcriptional activation.

4.4.3 The Smad3 linker region physically interacts with p300

We then determined whether the Smad3 linker region physically interacts with p300 by immunoprecipitation-immunoblot assay. Myc epitope-tagged Smad3 linker and HA epitope-tagged p300 were cotransfected into 293 cells, treated with or without TGF-ß. Cell lysates were then immunoprecipitated by the HA antibody. The immunoprecipitates were loaded onto a gel and detected by immunoblot using an antibody against the Myc epitope. As shown in Fig 4.3A and Fig 4.3B, the linker region interacts with p300-HA constitutively, in contrast to the TGF- β -induced interaction between the full length Smad3 and p300-HA. To provide further evidence for a physical interaction between the Smad3 linker region and p300, we analyzed whether GAL4-Smad3 (Linker) interacted with p300. GAL4-Smad3 (Linker) or GAL4-Smad3 (Full Length) were cotransfected with p300-HA into 293 cells, treated with or without TGF-B. Cell lysates were immunoprecipitated by the HA antibody, followed by immunoblot with an antibody against the Smad3 liker region. Similar to results in Fig 4.3A and Fig 4.3B, the GAL4-Smad3 (Linker) interacted with p300-HA constitutively, whereas the GAL4-Smad3 (Full Length) interacted with p300-HA in a TGF-B-dependent manner (Fig 4.3C and data not shown). Taken together, these results indicate that the linker region of Smad3 physically interacts with p300.





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Figure 4.3 The linker region of Smad3 physically interacts with p300.

(A), (B). 293 cells were cotransfected with Myc-Smad3 (Linker), Myc-Smad3 (Full Length), p300-HA, and TBRI (T204D) for TGF-B induction. Cell lysates were immunoprecipitated by HA antibody followed by immunoblot with an antibody against the Myc epitope. Expression levels of Myc-Smad3 (Linker) and Myc-Smad3 (Full Length) were examined by Myc immunoblot and p300-HA levels were examined by HA immunoblot. (C). 293 cells were cotransfected with GAL4-Smad3 (Linker), GAL4-Smad3 (Full Length), p300-HA, and TBRI (T204D) for TGF-B induction. Cell lysates were immunoprecipitated by HA antibody followed by immunoblot with an antibody against the Smad3 linker region. Expression levels of GAL4-Smad3 (Linker) and GAL4-Smad3 (Full Length) were examined by the antibody against Smad3 linker region. p300-HA levels were examined by HA immunoblot.

4.4.4 The linker region is necessary for native Smad3 to mediate TGF-β transcriptional activation responses

To analyze the role of the linker region in the native Smad3 protein, we generated a Smad3 (NC) construct with the linker region being deleted from the full length Smad3, leaving the N-terminal and C-terminal domains intact and connected with each other. To determine whether the Smad3 (NC) retained at least partial structural integrity, we analyzed its ability to be phosphorylated by the TGF-ß receptor at the C-tail and to form a heteromeric complex with Smad4. Smad3 (NC) or Smad3 (Full Length) were cotransfected with Smad4-HA into COS cells, treated with or without TGF-B. The lysates were directly analyzed by immunoblot with a phosphopeptide antibody that specifically recognized the receptor phosphorylated serine residues in the Smad3 C-tail. As shown in Figure 3.4A bottom panel, the C-tail of Smad3 (NC) was phosphorylated to approximately the same extent as that of Smad3 (Full Length). To analyze heteromeric complex formation with Smad4-HA, the lysates were immunoprecipitated with the HA antibody followed by immunoblot with an antibody that recognized the Smad3 Cterminal domain. As shown in Fig 4.4A top panel, Smad3 (NC) has a significantly increased ability than Smad3 (Full Length) to form a complex with Smad4.

The Smad3 (NC) was then analyzed for its ability to activate TGF-ß responsive reporter genes. As shown in Fig 4.4B, the Smad3 (NC) had little activity to stimulate transcription of three well-characterized TGF-ß responsive reporter genes: 3TP-Lux, Smad7-Lux, and A3-Lux. It even significantly inhibited the activation of the A3-Lux by endogenous Smads, possibly through sequestration of Smad4. Thus, the linker region of Smad3 is necessary for TGF-ß/Smads-mediated transcriptional activation.





4.4.5 The linker region and the C-terminal domain of Smad3 cooperate for transcriptional activation in the presence of TGF-B

Previous studies implied that the C-terminal domain of Smad3 is sufficient for maximal transcriptional activation upon TGF-β treatment. However, the Smad3 C-terminal domain frequently used in previous studies in fact contained one third of the linker region (For example, see refs 38 and 40). The results above indicated that the Smad3 linker region is necessary for activation of TGF-β responsive genes. We therefore determined whether the linker region and the C-terminal domain of Smad3 cooperate with each other for transcriptional activation. Expression plasmids encoding GAL4 fusions containing Smad3 linker region, Smad3 C-terminal domain, or both the linker region and the C-terminal domain, or both the linker region and the C-terminal domain of Smad3 synergize for transcriptional activation in the presence of TGF-β treatment. Immunoblot with an antibody against the GAL4 DNA binding domain confirmed that the three GAL4 fusion proteins were expressed at comparable levels (Fig 4.5B).

Figure 4.4 The linker region is necessary for native Smad3 to mediate TGF-ß transcriptional activation responses.

⁽A). Smad3 (NC) can be phosphorylated by the TGF-β receptor at the C-tail and has a significantly increased ability to form a heteromeric complex with Smad4. COS cells were cotransfected with Smad3 (NC) or Smad3 (Full Length) together with Smad4-HA and TβRI (T204D) for TGF-β induction. Cell lysates were analyzed for Smad3 C-tail phosphorylation and heteromeric complex formation with Smad4 as indicated. Expression levels of Smad3 (NC), Smad3 (Full Length), and Smad4-HA are also shown.
(B). Smad3 (NC) is unable to mediate TGF-β transcriptional activation responses. HepG2 cells were cotransfected with 3TP-Lux, Smad7-Lux, or A3-Lux plus FAST-1 together with the CS2 vector, Smad3 (Full Length), or Smad3 (NC), then treated with or without TGF-β, and analyzed for luciferase activity.



Figure 4.5 The linker region and the C-terminal domain of Smad3 synergize for TGF-βinduced transcriptional activation.

(A). COS cells were cotransfected with GAL4-Smad3 fusions containing the linker region, the C-terminal domain, or both the linker and C-terminal domain along with the GAL4 reporter gene and TBRI (T204D) for TGF-B induction. Cells were analyzed for luciferase activity.

(**B**). GAL4 protein expression levels were analyzed by immunoblot with the antibody against the GAL4 DNA binding domain.

(C). Schematic diagram for the structure and amino acids numbers for the Linker, C-terminal domain, and both Linker and C-terminal domain of Smad3.

4.4.6 Phosphorylation of Smad3 by CDK and ERK inhibits its interaction with p300

Since CDK and ERK phosphorylate several sites in the Smad3 linker region as well as the N domain, and the linker region can directly interact with p300, we asked the question whether phosphorylation of Smad3 by CDK and ERK affect the interaction of Smad3 with p300. Wild type Smad3 and a mutant Smad3 with all the CDK and ERK sites mutated to nonphosphorylatable Alanine or Valine [Smad3 (T8V/Linker Mut)] were co-transfected into 293 cells together with p300/HA. 48 hours after transfection, cells were treated with or without TGF- β and harvested. The interaction of wild type and mutant Smad3 with p300 were analyzed by immunoprecipitation with HA affinity beads followed by western blot with Smad3 antibody (Figure 4.6). The Smad3 which has all CDK/ERK sites mutated had significantly higher ability to interact with p300 than the wild type Smad3.

4.5 DISCUSSION

We have shown in this report that the Smad3 linker region has a transcriptional activity. Accordingly, Smad3 plays an important role in transcriptional control of a number of target genes. Smad3 and Smad2 have overlapping as well as distinct functions [157]. They are highly conserved in the N-terminal and C-terminal domains, but they differ in the linker region [1, 4, 156-160]. Whether the linker region of Smad2 also contains a transcriptional activation domain is not clear. We have generated a GAL4-Smad2 (Linker) plasmid, constructed in a very similar way as the GAL4-Smad3 (Linker). The resulting fusion had little transcriptional activity. However, this is complicated by the fact that the expression level of the GAL4-Smad2 (Linker) was very



Figure 4.6 Mutation of Smad3 CDK/ERK sites increases the ability of Smad3 to interact with p300

293 cells were co-transfected with wild type Smad3 or Smad3 (T8V/Linker Mut, in which the linker S/T-P sites, Thr 179, Ser 204, Ser 208, and Ser 213, as well as Thr 8 in the N domain were mutated to non-phosphorylatable Alanine or Valine) together with p300/HA. 48 hours after transfection, cells were treated with or without 500 pM TGF- β for 1 hour and harvested in TNM buffer, which contains 10 mM Tris-HCl pH 7.8, 150 mM NaCl, 3 mM MgCl₂, 0.5% NP-40. The lysates were immunoprecipitated by α HA affinity beads and the immunoprecipitates were analyzed by western blot with an antibody against Smad3.

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low (data not shown). Thus far, it is not clear why the expression of the GAL4-Smad2 (Linker) was low. Future studies are necessary to determine whether the Smad2 linker region also contains a transcriptional activation domain.

We have also analyzed whether the linker region plays an important role in the activation of several TGF-B/Smad-responsive reporter genes. We generated a Smad3 (NC) construct that contains only the N-terminal and C-terminal domains. The Smad3 (NC) has little activity to stimulate transcription of TGF-B responsive reporter genes. Since the Smad3 (NC) can be phosphorylated by the TGF- β receptor and has a significantly increased ability to form a heteromeric complex with Smad4, it is likely that the lacking of transcriptional activity in the Smad3 (NC) is due to the removal of a necessary activation function in the linker region. Introduction of Smad3 (NC) has distinct effects on different TGF-B responsive promoters (Fig 4.4B), which may be due to different configurations of binding sites for Smads and for other transcription factors. The Smad3 (NC) markedly inhibited the activation of A3-Lux by endogenous Smads. This may occur through competition with endogenous Smad2 and Smad3 for formation of heteromeric complexes with Smad4. In addition, we made a Smad3 (NL) construct, which encodes only the N-terminal domain and the linker region. The Smad3 (NL) has a very low activity to stimulate transcription of several TGF-B/Smad-responsive reporter genes we analyzed (data not shown). Smad3 functions as an oligomer, often as a homotrimer at basal state and in a heterotrimeric complex with Smad4 after TGF-B treatement [70]. Although the Smad3 (NL) construct contains the N-terminal domain for DNA binding and an activation domain in the linker region, it lacks the oligomerization

motifs that reside within the C-terminal domain, which provides an explanation for the very low activity of the Smad3 (NL) construct.

We have shown that the linker and the C-terminal domain of Smad3 cooperate for transcriptional activation in the presence of TGF-B. The C-terminal domain was previously shown to have transcriptional activity. It is worth pointing out that the Cterminal domain of Smad3 used in the previous studies often contains one third of the linker region, amino acids 199-230 (For example, see refs 38 and 40). As shown in this report, the C-terminal domain alone has a lower activity when compared with the linker and the C-terminal domain together. How the linker region and the C-terminal domain of Smad3 synergize in the presence of TGF- β is not clear. In a related study, the crystal structure of a Smad4 fragment containing the SAD and the C-terminal domain has been solved [72]. The C-terminal domain of Smad4 is highly homologous with that of Smad2 and Smad3 (50% identity), except that Smad4 has a unique insert of \sim 35 amino acids which interacts with the C-terminal tail to form a TOWER-like structural extension from the core. The crystal structure suggests that SAD provides transcriptional capability by reinforcing the structural core and coordinating with the TOWER to present the prolinerich surface and a glutamine-rich surface in the TOWER for interaction with transcription partners [72]. It remains to be determined whether the linker region of Smad3 exerts transcriptional activity through a similar mechanism. Since both the linker region and the C-terminal domain of Smad3 can interact with p300, it is possible that presentation of certain surfaces in the linker region and the C-terminal domain engages p300 in an optimal conformation for transcriptional activation.

The Smad3 linker region is proline- and serine-rich. It contains demonstrated as well as suspected phosphorylation sites for multiple kinases, such as the cyclin-dependent kinases, ERK mitogen-activated protein (MAP) kinase, c-Jun N-terminal kinase, p38 MAP kinase, and Ca²⁺-calmodulin-dependent kinase II [1, 48, 50, 144, 145, 149, 151]. Phosphorylation of the linker region by the various kinases may differentially influence Smad3 transcriptional activity in a context-dependent manner. The C-terminal domain of Smad3 is regulated by TGF-ß receptor phosphorylation [1, 4, 156-160]. The C-terminal domain of Smad3 is also a protein-protein interaction domain, responsible for homotrimerization, hetero-trimerization with Smad4, and also interaction with a number of DNA binding proteins [1 4, 70, 156-160]. Under different conditions, the linker region and the C-terminal domain may have varying transcriptional activities, leading to distinct biological responses.

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