Protein Acetylation –
A Multifunctional Regulator of TGF-β Signaling

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Abstract

Transforming growth factor β (TGF-β) is a member of a large family of cytokines that regulate many crucial events in cells, including proliferation, differentiation, migration and apoptosis. Deregulated TGF-β signaling is associated with various forms of cancers and developmental disorders. TGF-β binds to a receptor complex at the surface of cells and activates a signaling cascade involving specific intracellular signaling proteins, known as Smads. Following receptor activation, the Smads are activated by phosphorylation and translocate to the nucleus, where they activate or repress the expression of specific genes.

Posttranslational modifications regulate the function of proteins in a number of ways, including their activity, stability, localization, and/or interactions with other proteins. These modifications are important to modulate the strength and specificity of cellular signal transduction. Smad7, an important negative modulator of TGF-β signaling, has been shown to be acetylated by the acetyltransferase p300. My aim was to further explore the involvement of protein acetylation in TGF-β-dependent signaling.

We could show that the acetylation of Smad7 is a reversible process. Interestingly, earlier work had shown that the acetylation of Smad7 prevented its degradation. In agreement with this observation, we found that the ubiquitylation and degradation of Smad7 was increased following cotransfection with HDAC1, a protein deacetylase. Based on our observations, we propose a model in which the stability of Smad7 is controlled by the balance between its acetylation, deacetylation and ubiquitylation. In a separate study, we found that also Smad2 and Smad3 are acetylated by p300/CBP and P/CAF upon TGF-β stimulation. Moreover, we found that the acetylation of the short isoform of Smad2 promoted its DNA binding activity, resulting in an increased transcriptional activity. Our results suggest that the increased DNA binding in response to acetylation is due to a conformational change in Smad2. During the course of this work, we found that Smad2 and Smad7 have intrinsic acetyltransferase activity. This is the first time that Smad proteins are shown to have enzymatic activity, and our results suggest that the acetyltransferase activity of Smad2 represents a novel way to regulate TGF-β signaling.

Keywords: TGF-β, Acetylation, Deacetylation, Acetyltransferase, HDAC, Smad, Transcription

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Till Per
& min familj
Publications

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:


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Related Publications


*Det högsta är ej att aldrig falla, utan att resa sig efter varje fall.*

*kinesiskt ordspråk*
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## Abbreviations

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<tbody>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>Co-Smad</td>
<td>Common mediator Smad</td>
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<tr>
<td>EMSA</td>
<td>Electromobility shift assay</td>
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<td>HAT</td>
<td>Histone acetyltransferase</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<td>I-Smad</td>
<td>Inhibitory Smad</td>
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<td>MH1/2</td>
<td>Mad homology domain 1/2</td>
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<tr>
<td>NES</td>
<td>Nuclear export signal</td>
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<td>NLS</td>
<td>Nuclear localization signal</td>
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<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>P/CAF</td>
<td>p300/CBP associated factor</td>
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<tr>
<td>R-Smad</td>
<td>Receptor-activated Smad</td>
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<tr>
<td>SBE</td>
<td>Smad binding element</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SMURF</td>
<td>Smad ubiquitylation regulatory factor</td>
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<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<tr>
<td>TβR</td>
<td>TGF-β receptor</td>
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<tr>
<td>TSA</td>
<td>Trichostatin A</td>
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Introduction

It is all about timing, being at the right place at the right time and doing the right thing. All proteins in a cell have to be in their correct positions at the right time to perform their specific functions. Cells achieve this through elaborate signaling systems, relying on a continuous flow of information, not only between cells and their environment, but also within cells. If these signaling pathways are perturbed, it may cause either pathological conditions or developmental defects. Cells communicate by either direct cell-cell contacts, interactions with extracellular matrix components or by secreted soluble signaling factors. These factors, including neurotransmitters, hormones, cytokines and growth factors, bind to specific receptors at the plasma membrane. When these factors bind to their receptors, the receptors become activated and the external signal is converted to an intracellular signal. These signaling pathways regulate a diverse set of cellular events, such as survival, cell division, cell death, movement or differentiation.

Transforming growth factor β (TGF-β) is a member of a large family of cytokines that regulate a diverse set of events in cells, including proliferation, differentiation, migration and apoptosis. TGF-β exerts these functions by binding to specific receptors at the plasma membrane. These receptors then activate a family of transcription factors known as Smads, which regulate the expression of specific genes.

Within a specific signaling pathway, the function of individual proteins is regulated by posttranslational modifications in order to produce accurate signals. The most common modifications are phosphorylation, glycosylation, sumoylation, acetylation and ubiquitylation. These modifications regulate the activity, stability, localization, or interactions between proteins. Although the TGF-β pathway has been studied extensively, the role of acetylation of proteins in this pathway was still unexplored. Therefore, my aim has been to study the direct involvement of protein acetylation in TGF-β-dependent signal transduction.
Transforming growth factor β (TGF-β)

TGF-β is a member of a large family of cytokines that regulate key cellular processes, including cell proliferation, differentiation and apoptosis, both during embryogenesis and in mature tissues (Heldin, et al. 1997; ten Dijke, et al. 2000; Shi, et al. 2003). TGF-β1 was discovered approximately 25 years ago and named based on its ability to induce the growth of normal rat kidney fibroblasts in soft agar assays (Roberts, et al. 1981; Anzano, et al. 1983). Later, it turned out that TGF-β was a potent growth inhibitor and a multifunctional protein (Dennler, et al. 2002). TGF-β exerts its functions in a wide variety of cell types. TGF-β inhibits proliferation of epithelial, endothelial and haematopoietic cells. In addition, it regulates the differentiation of immune, neuronal, mesenchymal and epithelial cell types and modulates their apoptotic response (Moustakas, et al. 2002).

The TGF-β family consists of more than 60 characterized structurally related members, with 34 proteins encoded in the human genome (Feng, et al. 2005). This large family, where TGF-β serves as a prototype, can be subdivided into three general classes, i.e. TGF-β, activins, and BMPs (bone morphogenetic proteins), based on structural and functional criteria (Cohen 2003). There are three TGF-βs, five activins and at least eight BMPs, all encoded by different genes (Feng, et al. 2005). The signaling pathways involving the TGF-β family are well conserved among all eukaryotic organisms (Attisano, et al. 2002).

TGF-β ligands and receptors

Three isoforms of TGF-β have been identified in mammals, TGF-β1 (Derynck, et al. 1985), TGF-β2 (de Martin, et al. 1987) and TGF-β3 (Derynck, et al. 1988; ten Dijke, et al. 1988). The formation of homodimers is most common, but heterodimers between different TGF-β isoforms have also been reported (Cheifetz, et al. 1987; Ogawa, et al. 1992). The isoforms show high sequence similarity and are highly similar in their biological activities in vitro. However, they display different expression patterns and functions in vivo (Roberts, et al. 1992). Thus, mutants of the three isoforms show distinct and only partially overlapping phenotypes (Dunker, et al. 2000).
Because of the important biological roles of TGF-β family members, it is necessary to regulate their activity. Thus, TGF-β is synthesized as an inactive precursor that needs to be activated by proteolytic cleavage by more than one protease in order to be active. The activation process also involves formation of complexes that ensure the correct folding of TGF-β and enhances its stability and secretion to the extracellular matrix (Koli, et al. 2001; Lawrence 2001). Furthermore, TGF-β ligands associate with several extracellular proteins that modulate their activities. For example, the extracellular matrix proteoglycans decorin and biglycan bind and inhibit TGF-β (Yamaguchi, et al. 1990).

TGF-β signaling is initiated by the binding of the ligand (TGF-β) to a heterotetrameric complex of two types of transmembrane serine/threonine kinases, the TGF-β type I (TβRI) and type II (TβRII) receptors (Derynck, et al. 1997; Heldin, et al. 1997; Massagué 1998). Activation of the receptors triggers a signaling cascade involving specific intracellular signaling proteins, so called Smads. Following receptor activation, the Smads are activated by phosphorylation and translocate to the nucleus, where they activate or repress specific target genes (Figure 1) (Heldin, et al. 1997; Zhang, et al. 1999; Massagué, et al. 2000; Attisano, et al. 2002).

![Figure 1. Schematic illustration of the TGF-β signaling pathway.](image-url)
The TGF-β receptor family is divided into two subfamilies, the type I and type II receptors, based on their structural and functional properties. Five type II receptors and seven type I receptors have been identified in mammals (Derynck, et al. 1997; Massagué 1998). The nomenclature of the type I receptors is complex, since they were cloned simultaneously by different groups. One practice has been to name them ALK (activin receptor-like kinase) 1 to 7 (Massagué 1998). When their ligands became known, the receptors were given more descriptive names. Thus, the TGF-β type I receptor originally known as ALK5 (Franzén, et al. 1993), is now called TβR-I (Yamashita, et al. 1994).

The TGF-β receptors are transmembrane glycoproteins that are structurally related. They contain an extracellular N-terminal domain which binds the ligand, a transmembrane domain and a C-terminal serine/threonine kinase domain (Heldin, et al. 1997; Massagué 1998). The type II receptor kinase is constitutively active and forms a complex with the type I receptor following ligand binding (Wrana, et al. 1994; Wieser, et al. 1995). As a result, the type II receptor phosphorylates and activates the type I receptor. The phosphorylation occurs on serine and threonine residues in a highly conserved glycine-serine-rich region (termed the GS domain), which is close to the kinase domain of the type I receptor. The GS domain is a key regulatory region that controls the kinase activity of the type I receptor. Phosphorylation of the GS domain changes the conformation of the type I receptor, thereby activating its kinase activity (Figure 2) (Wrana, et al. 1994; Franzén, et al. 1995; Wieser, et al. 1995; Souchelnytskyi, et al. 1996). Thus, the type I receptors act downstream of the type II receptors and determine the specificity of the signal (Attisano, et al. 2002).

The L45 loop is a region within the kinase domain of the type I receptor that determines which Smads that will bind and become phosphorylated by the receptor complex (Sekelsky, et al. 1995; Feng, et al. 1997; Persson, et al. 1998). The structure of the L45 loop differs between receptors with different signaling specificities. Receptors regulating the TGF-β-activated Smads, Smad2 and Smad3, have a conserved L45 loop, while receptors activating the BMP-activated Smads, Smad1, Smad5 and Smad8, have slightly different L45 loops (Feng, et al. 1997; Chen, et al. 1998). In addition, the GS domain also contributes to the binding of Smads (Wu, et al. 2001).

The diversity in TGF-β signaling is achieved by the formation of complexes between specific type I and type II receptors, to which only a distinct set of TGF-β ligands can bind. Related ligands often bind to the same receptor complexes. The large number of TGF-β ligands enables cells to fine-tune the signaling pathway, and the expression patterns of the various ligands are therefore of great importance (Feng, et al. 2005). A third group of TGF-β receptors, the accessory receptors, also contributes to the specificity of the system. These receptors, including betaglycan, endoglin and cripto, promote receptor activation by either increasing the concentration of ligand close to
the signaling receptors or by promoting binding of the ligand to the signaling receptors (Shi, et al. 2003). The function of so called “ligand traps” are opposite to that of the accessory receptors. As the name implies, these proteins prevent binding of the ligand to the receptor. For example, α2-macroglobulin and decorin both bind free TGF-β and inhibit signal transduction. Several ligand traps for BMPs have also been described (Massague, et al. 2000; Balemans, et al. 2002). Furthermore, inhibitory proteins can bind to the type I receptor and regulate its activity. One example of this is the binding of FKBP12 to the unphosphorylated GS domain of the type I receptor, which prevents signaling (by the type I receptor) in the absence of ligand (Huse, et al. 1999; Wang, et al. 2004). In contrast, disabled-2 promotes TGF-β signaling by stabilizing the interaction between Smad2/3 and the receptor complex (Hocevar, et al. 2001).

![Schematic illustration of TGF-β-induced activation of the receptor-complex and R-Smads.](image-url)

**Figure 2.** Schematic illustration of TGF-β-induced activation of the receptor-complex and R-Smads.
Smad proteins

Smad proteins are intracellular mediators of the TGF-β signal. Smads were first identified in genetic screens in worms and flies, and the name Smad is a fusion of the names from *C. elegans* (Sma) and *Drosophila* (Mad) (Massagué 1998). Eight mammalian Smads have been described and they are ubiquitously expressed throughout development and in all adult tissues (Moustakas, et al. 2001). Functionally, Smads fall into three subfamilies, i.e. receptor-activated Smads (R-Smads; Smad1, Smad2, Smad3, Smad5 and Smad8), common mediator Smad (Smad4) and inhibitory Smads (I-Smads; Smad6 and Smad7) (Attisano, et al. 2002). Receptor-activated Smads can be further divided into two subgroups depending on the signals they mediate; Smad2 and Smad3 are involved in TGF-β/activin signaling and are activated by activin, nodal and TGF type I receptors (ALK-4, -5 and-7), while Smad1, Smad5 and Smad8 are referred to as BMP-activated R-Smads and are activated by BMP type I receptors (ALK-2, ALK-3 and ALK-6) and by ALK-1 (Miyazawa, et al. 2002).

R-Smads and Smad4 share two highly conserved domains, Mad-homology domains 1 and 2 (MH1 and MH2), located in the N- and C-terminal parts of the proteins, respectively. These two domains are connected by a divergent proline-rich region of variable length, usually referred to as the linker region (Massagué 1998; Shi 2001) (Figure 3). The MH2 domain is conserved in all three subclasses of Smads, whereas the MH1 domain is conserved in the R-Smads and Smad4, but not in the I-Smads. The MH2 domain of the R-Smads is responsible for receptor interaction, transactivation and oligomerization. In addition, the MH2 domain is involved in interactions with other proteins and in the nuclear transport of Smads (Massagué 1998; Shi, et al. 2003). The MH1 domain of Smad3, Smad4 and the short isoform of Smad2 exhibits sequence-specific DNA binding activity. This domain may also play a role in nuclear import, and it negatively regulates the function of the MH2 domain (Shi 2001; Shi, et al. 2003). The linker region is divergent among the Smads and contains multiple phosphorylation sites (Kretzschmar, et al. 1997; Kretzschmar, et al. 1999; Derynck, et al. 2003). The interaction with Smurfs (HECT-domain-containing E3 ubiquitin ligases) occurs through a PY motif found in the linker region (Zhu, et al. 1999; Kavsak, et al. 2000; Ebisawa, et al. 2001; Zhang, et al. 2001).
R-Smad activation

The R-Smads contain a characteristic SSXS motif in their extreme C termini and this motif is phosphorylated by type I receptors upon ligand binding, resulting in activation of the R-Smads (Macias-Silva, et al. 1996; Abdollah, et al. 1997; Souchelnytskyi, et al. 1997). The substrate specificity is controlled by the L45 loop of the type I receptor that recognizes the L3 loop in the MH2 domain of Smads (Chen, et al. 1998; Lo, et al. 1998). Although Smad2/3 and Smad1/5/8 only differ by two amino acids in their L3 loops, this difference is sufficient for the type I receptors to discriminate between the different R-Smads (Chen, et al. 1998; Lo, et al. 1998).

The R-Smads need to come in close proximity of the activated type I receptor to be phosphorylated and activated. Several adaptor proteins are known to support the presentation of the R-Smads to the activated receptor complex. The best characterized adaptor protein is SARA (Smad anchor for receptor activation) (Tsukazaki, et al. 1998). SARA is a FYVE-domain-containing membrane-bound protein that can regulate the subcellular localization of non-activated R-Smads by sequestering them in the cytoplasm (Xu, et al. 2000). Another crucial function of SARA is to present R-Smads to the activated type I receptor (Tsukazaki, et al. 1998). SARA interacts with Smad2 and Smad3, but not with Smad1, -5, -8, and therefore confers specificity to TGF-β signaling (Tsukazaki, et al. 1998). Structural studies show that SARA contains a Smad-binding domain, SBD, which interacts with the MH2 domains of specific Smads (Wu, et al. 2000). The FYVE domain of SARA is important for its localization to early endosomes, where it interacts with both Smads and receptors (Itoh, et al. 2002). It is still controversial whether endocytosis is necessary for TGF-β signaling. It was demonstrated that the transcriptional activity of a Smad2-responsive promoter was reduced when endocytosis was inhibited (Hayes, et al. 2002; Runyan, et al. 2005).
However, other reports indicate that endocytosis is not required for TGF-β signaling (Goto, et al. 2001; Lu, et al. 2002).

Hgs is another FYVE-containing adaptor protein that has been suggested to cooperate with SARA to facilitate recruitment of Smad2 and Smad3 to the receptor (Miura, et al. 2000). Like SARA and Hgs, ELF is important for the recruitment of Smad3 and Smad4 to the receptor by controlling their subcellular localization (Mishra, et al. 2006). In addition, it has been reported that the microtubuli system sequesters inactivate R-Smads in the cytoplasm, thereby negatively regulating TGF-β signaling (Dong, et al. 2000).


Smad4 plays a central role in both TGF-β/activin and BMP signaling, since it forms complexes with R-Smads activated by all TGF-β ligands. Only one form of Smad4 has been identified in mammals, while two have been found in Xenopus laevis (Howell, et al. 1999; Masuyama, et al. 1999). However, it was recently found that TIF1γ (transcriptional intermediary factor 1γ), similarly to Smad4, binds R-Smads and mediates different transcriptional effects. The interaction with TIF1γ is specific for Smad2 and Smad3 and is TGF-β-dependent (He, et al. 2006). This implies that Smad signaling may be more diverse than previously thought. The exact role of TIF1γ is still controversial, since one group claims that TIF1γ competes with Smad4 for binding to the R-Smads, implying that Smad4 is not the only partner for Smads, while another group has suggested that TIF1γ exerts its function by regulating the stability of Smad4, since it has ubiquitin ligase activity (Dupont, et al. 2005; He, et al. 2006; Heldin, et al. 2006).

**Nuclear shuttling of Smads**

The subcellular transport of Smads between the cytoplasm and the nucleus confers an additional level of regulation to TGF-β signaling. In unstimulated cells, R-Smads are predominantly cytoplasmic, while Smad4 is distributed throughout the cytoplasm and nucleus. Upon ligand stimulation, both types of Smads rapidly accumulate in the nucleus (Reguly, et al. 2003). Both R-Smads and Smad4 contain nuclear localization signals in their MH1 domains. Activation of the R-Smads by phosphorylation results in a conformational change in the R-Smads that may expose their nuclear localization sig-
nals, thereby facilitating interactions with proteins involved in nuclear import (Reguly, et al. 2003).

The nuclear localization signal in Smad3 has been shown to interact with importin β1, which transports Smad3 into the nucleus (Xiao, et al. 2000). In contrast, Smad2 is unable to interact with importin β1. Instead, Smad2 interacts with the nucleoporins CAN/Nup214 and Nup153, which mediates its nuclear transport (Xu, et al. 2002). Moreover, both Smad3 and Smad4 have been shown to interact with CAN/Nup214, which is sufficient for their nuclear import (Xu, et al. 2003).

The R-Smads constantly shuttle between the cytoplasm and the nucleus during active signaling. The R-Smads become dephosphorylated in the nucleus by the newly discovered phosphatase PPM1A and shuttle back to the receptor to take part in another round of signaling (Lin, et al. 2006). The constant shuttling of the R-Smads during active signaling guarantees that signaling occurs only when the receptor is active (Inman, et al. 2002). Moreover, kinetic analyzes demonstrate that the increased levels of Smad2 in the nucleus during TGF-β stimulation is caused by a decrease in its nuclear export, due to selective nuclear trapping of phosphorylated Smad2. The predominantly cytoplasmic localization of Smad2 in unstimulated cells is explained by the fact that the export of Smad2 is more efficient than its import (Schmierer, et al. 2005).

I-Smads and their function

The third group of Smads, the I-Smads (Smad6 and Smad7), are negative regulators of TGF-β signaling. Interestingly, the expression of Smad6 and Smad7 is enhanced by TGF-β and BMP, resulting in negative feedback regulation of the Smad signaling pathway (Nakao, et al. 1997; Afrakhte, et al. 1998). Smad6 and Smad7 appear to have overlapping functions, but Smad6 preferentially inhibits BMP signaling, while Smad7 inhibits both TGF-β and BMP signaling (Massagué 1998). Smad-binding elements are found in the promoters of both Smad6 and Smad7. Both Smad3 and Smad4 are recruited to the Smad7 promoter and this is important for the expression of the Smad7 gene in response to TGF-β signaling (Nagarajan, et al. 1999; Brodin, et al. 2000). The expression of the Smad7 gene is also regulated by other growth factors and cytokines, such as EGF, IFNγ and tumor necrosis factor, suggesting crosstalk between different signaling pathways (Derynck, et al. 2003).

Smad6 and Smad7 inhibit TGF-β signaling in several ways (Figure 4). It has been demonstrated that both proteins associate with activated type I receptors, thereby preventing phosphorylation of R-Smads (Imamura, et al. 1997; Nakao, et al. 1997). In addition, it has been demonstrated that Smad6 interacts with phosphorylated Smad1, thereby preventing the interaction between Smad1 and Smad4 (Hata, et al. 1998). Furthermore, Smad7 inter-
acts with the ubiquitin ligases Smurf1 and Smurf2 in the nucleus. In response to TGF-β stimulation, the Smad7-Smurf complex translocates from the nucleus to the plasma membrane, where the Smurfs promote the ubiquitylation and degradation of the receptor complex (Kavsak, et al. 2000; Ebisawa, et al. 2001). WWP1, another ubiquitin ligase structurally related to Smurfs, also interacts with Smad7 to inhibit TGF-β signaling (Komuro, et al. 2004). Finally, Smad7 interacts with GADD34 (growth arrest and DNA damage protein 34), a regulatory subunit of the phosphatase PP1. Smad7 acts as an adaptor molecule and recruits GADD34/PP1 to the receptor, which results in dephosphorylation and inactivation of the receptor (Shi, et al. 2004).

Figure 4. I-Smads use several mechanisms to regulate TGF-β signaling.

Smad7 resides in the nucleus of unstimulated cells and translocates to the plasma membrane following receptor activation (Itoh, et al. 1998). I-Smads lack SSXS motifs and are not phosphorylated by the TGF-β receptor in their C termini. However, Smad7 is phosphorylated on serine-249 by an unknown kinase. This phosphorylation is important for its ligand-independent ability to regulate transcription (Pulaski, et al. 2001). Furthermore, several studies demonstrate a direct involvement of Smad6 and Smad7 in transcriptional regulation, adding another possibility to regulate TGF-β signaling. I-Smads can regulate transcription by interacting with corepressors/coactivators, as
Posttranslational modifications of Smads

Posttranslational modifications of proteins confer additional regulatory possibilities. Modifications such as phosphorylation, acetylation, methylation, sumoylation and ubiquitylation are the most common modifications. These modifications regulate protein-protein interactions, protein-DNA interactions, as well as protein localization and stability (Kouzarides 2000). Polyubiquitylation of proteins by ubiquitin ligases is of major importance for regulating protein stability (Ciechanover, et al. 2004).

Phosphorylation and ubiquitylation are the most studied modifications associated with TGF-β signaling. As already mentioned, the receptor complex is ubiquitylated by Smurf1, Smurf2, WWP1 and NEDD4-2 and thereby targeted for proteosomal degradation (Kavsak, et al. 2000; Ebisawa, et al. 2001; Komuro, et al. 2004; Kuratomi, et al. 2005). Furthermore, R-Smads, as well as Smad4 and I-Smads, are also ubiquitylated by different ubiquitin ligases. Smad2 has been shown to become ubiquitylated by Smurf2, NEDD4-2 and Tiu1 (Zhang, et al. 2001; Seo, et al. 2004; Kuratomi, et al. 2005). Smad2 and Smad3 show high sequence similarity. Despite this, they are ubiquitylated by different ubiquitin ligases. A complex consisting of ROC1, Skip1, Cullin1 and Fbw1a induces the ubiquitylation of Smad3 (Fukuchi, et al. 2001). Smad4 is targeted for ubiquitylation by a number of ubiquitin ligases, including Smurf1, Jab1, SCFβ-TrCP1 and CHIP (Wan, et al. 2002; Li, et al. 2004; Wan, et al. 2004; Morén, et al. 2005). Moreover, Smad4 can be mono-ubiquitylated and sumoylated, which increases its stability and thereby enhances TGF-β signaling (Lee, et al. 2003; Morén, et al. 2003; Ohshima, et al. 2003; Liang, et al. 2004). However, it has also been shown that sumoylation of Smad4 can lead to inactivation of nuclear Smad signaling (Long, et al. 2004).

It has been demonstrated that Smad7 interacts with the coactivator p300 and becomes acetylated on lysines 64 and 70. This acetylation protects Smad7 from Smurf-mediated degradation (Gröñoos, et al. 2002). Smad7 can be ubiquitylated by both Smurf1 and Smurf2 (Kavsak, et al. 2000; Ebisawa, et al. 2001). In addition, it has been shown that Arkadia promotes polyubiquitylation and degradation of Smad7 (Koinuma, et al. 2003). Most
likely, this is only the tip of the iceberg and more modifications of molecules involved in TGF-β signaling will be discovered in the near future.

**Physiological roles of TGF-β**

TGF-β members mediate many key events during development, as well as in tissue homeostasis, and have diverse effects on a wide variety of cellular responses, including cell cycle control, apoptosis, angiogenesis, chemotaxis, immune function and extracellular matrix formation (Whitman 1998; Piek, *et al.* 1999; Massagué, *et al.* 2000; Dennler, *et al.* 2002; Cohen 2003). TGF-β is a potent inhibitor of proliferation in many cell types, including epithelial, endothelial and hematopoietic cells, and regulates the differentiation of immune, neuronal, mesenchymal and epithelial cells, as well as the apoptotic response of certain cell types (Moustakas, *et al.* 2002).

The growth inhibitory activity of TGF-β involves the transcriptional control of key regulators of the cell cycle. The transcriptional regulation involves both activation and repression of genes important for cell cycle progression (Moustakas, *et al.* 2002). TGF-β inhibits proliferation by arresting cells primarily in the G1 phase of the cell cycle (Laiho, *et al.* 1990).

TGF-β has been demonstrated to enhance the expression of the cell cycle inhibitors p15 and p21 (Hannon, *et al.* 1994; Datto, *et al.* 1995; Pardali, *et al.* 2000), and repress the expression of certain cell cycle activators, including c-Myc and Id proteins (Pietenpol, *et al.* 1990; Ling, *et al.* 2002; Kowanetz, *et al.* 2004). The cyclin-dependent kinase inhibitors p15 and p21 inhibit the phosphorylation of the retinoblastoma protein, Rb, by inhibiting the activity of CDK4/6-cyclin D and CDK2-cyclin E complexes. Hypo-phosphorylated Rb is unable to release the transcription factor E2F, and transcription of genes regulated by E2F is therefore repressed (*Figure 5*). Genes regulated by E2F are crucial for the progression of the cell cycle towards S-phase (Massagué, *et al.* 2000; Massague, *et al.* 2006).

The transcription factor c-Myc promotes cell growth and proliferation. It has been shown that Smads down-regulate the expression of c-Myc by binding to its promoter together with E2F4/5 and p107 (Chen, *et al.* 2002). c-Myc has the ability to both activate and repress the expression of target genes; it induces the expression of cyclin D2 and CDK4 (Bouchard, *et al.* 1999; Hermeking, *et al.* 2000), and represses the expression of p15 and p21 (Claassen, *et al.* 2000; Staller, *et al.* 2001). Since cyclin D2 and CDK4 are important for cell cycle progression, and p15 and p21 repress this process, down-regulation of c-Myc by TGF-β inhibits cell cycle progression.

TGF-β down-regulates the expression of Id1, Id2 and Id3 (Ling, *et al.* 2002; Kowanetz, *et al.* 2004). Id proteins are negative regulators of cell differentiation. They regulate differentiation by inhibiting the DNA binding activity of certain basic helix-loop-helix transcription factors. Many of these
transcription factors are positive regulators of differentiation. Id proteins also interact with Rb and therefore promote cell proliferation (Norton 2000; Siegel, et al. 2003; Sikder, et al. 2003).

Figure 5. Schematic illustration of the involvement of TGF-β in cell cycle regulation.

In addition to inhibiting growth, TGF-β also induces apoptosis in a cell type and context dependent manner. TGF-β can regulate apoptosis both in a Smad-dependent and -independent manner (Dennler, et al. 2002). TGF-β has been found to regulate the expression of both pro- and anti-apoptotic Bcl family members, which are important regulators of apoptosis (Motyl, et al. 1998; Francis, et al. 2000). Moreover, TGF-β has also been shown to activate TAK-1 (TGF-activated kinase 1), a protein belonging to the MAPK kinase family. TAK-1 can activate the p38 pathway, which is involved in regulating apoptosis (Yamaguchi, et al. 1995). It has also been proposed that Daxx, a Fas-receptor-associated protein, acts downstream of the TβR-1, resulting in activation of the JNK pathway and apoptosis (Figure 6) (Perlman, et al. 2001).
TGF-β is also an important regulator of the immune response, both the antigen-specific and the non-specific. TGF-β inhibits B- and T-cell proliferation and stimulates apoptosis, thus acting as an immunosuppressive molecule (Dennler, et al. 2002). It has also been shown that TGF-β plays a role in antibody class-switching, which is transcriptionally regulated via Smad binding elements, SBEs, found in the regulatory enhancer region close to the immunoglobulin Cα promoter and the human IgA genes (Hanai, et al. 1999; Pardali, et al. 2000).

The formation of new blood vessels can occur by two different mechanisms, vasculogenesis and angiogenesis. Angiogenesis is important for wound healing and tissue remodeling (Beck, et al. 1997), and TGF-β has been shown to be involved in both these processes (Pepper 1997). Angiogenesis is characterized by several different processes, such as degradation of the extracellular matrix, proliferation and directed migration of endothelial cells followed by lumen formation (Carmeliet 2000). In order to investigate the role of TGF-β in angiogenesis, several studies have been performed over the years both in vitro and in vivo. Some studies have suggested that TGF-β inhibits angiogenesis, most likely since it inhibits proliferation and migration of endothelial cells (EC). However, other studies suggested that TGF-β promoted angiogenesis (Pepper 1997). This discrepancy can be explained by more recent studies showing that TGF-β has a dual function in angiogenesis. This dual function can be explained by the expression of both Anti-apoptotic proteins and pro-apoptotic proteins.

**Figure 6.** Schematic illustration of the involvement of TGF-β in apoptosis. Adapted from Dennler et al., 2002.
ALK1 and ALK5 receptors in EC cells (Goumans, et al. 2003). ALK1 and ALK5 have been demonstrated to have opposite functions and TGF-β binds to both receptors. ALK5, which is expressed on most cell types, activates Smad2 and Smad3 and inhibits migration and proliferation of EC cells. ALK1, which is selectively expressed on EC cells activates Smad1,-5 and -8, resulting in the expression of Id1 which stimulates migration and proliferation, thereby promoting angiogenesis. Angiogenesis is therefore depending on the balance between the activities of ALK1 and ALK5 (Figure 7) (Goumans, et al. 2003).

![Diagram](image)

**Figure 7.** Illustration of the dual function of TGF-β in the regulation of angiogenesis. Adapted from Goumans et al., 2003.

The relationship between TGF-β and cancer is complex. TGF-β was first identified as a growth promoting factor, thereby its name. Later, TGF-β turned out to be a potent growth inhibitor, and was also shown to enhance metastasis formation and increase tumor invasiveness (Roberts, et al. 2003). TGF-β acts as a tumor suppressor in premalignant cells and also in cells progressing through the early stages of carcinogenesis. TGF-β achieves this mainly by promoting growth inhibition and apoptosis (Gold 1999; de Caestecker, et al. 2000). However, in the later phases of carcinogenesis, cells escape from the anti-mitogenic responses and start to utilize TGF-β for immunosuppression, angiogenesis and EMT, all in favor for tumor formation (Roberts, et al. 2003) (Figure 8). Furthermore, it is often observed that tu-
Tumor cells start to secrete high amounts of TGF-β and this correlates with the severity of the tumor. Thus, TGF-β contributes indirectly to tumor growth and invasiveness by affecting cells surrounding the tumor, by inducing angiogenesis, by suppressing the immune response, or by acting directly on the tumor cells (Siegel, et al. 2003). Epithelial to mesenchymal transition, EMT, is a complex process where epithelial cells acquire mesenchymal characteristics. EMT involves changes in cell morphology, reorganization of the cytoskeleton, alterations in epithelial cell junctions and increased motility, all in favor of invasiveness of cancer cells (Zavadil, et al. 2005). Several reports show that TGF-β is able to induce EMT, thereby promoting metastasis formation and invasiveness (Siegel, et al. 2003).

**Figure 8.** Schematic illustration of the dual function of TGF-β in cancer. Adapted from Roberts and Wakefield 2003.
Transcription factors

The fundamental dogma of molecular biology is that DNA is copied into RNA, which in turn is copied into proteins. The process in which RNA is produced from DNA is called transcription. Thus, transcription is the first step in the synthesis of new proteins. This makes it an attractive control point for regulating the expression of genes in particular cell types or in response to specific signals.

Transcription is a tightly regulated process involving many levels of regulation, including a plethora of specific DNA elements, proteins and protein complexes. RNA polymerase II is a multisubunit enzyme that catalyzes the synthesis of mRNA from a DNA template. It is one of the key components in gene expression and its regulation is of major importance. Other components involved in the regulation of gene expression include the core promoter, cis-acting sequences (including enhancers, silencers and proximal promoter regions), general transcription factors, sequence-specific DNA-binding transcription factors and cofactors (Butler, et al. 2002).

General/basal transcription factors (GTFs) are required to initiate transcription. GTFs promote the formation of the preinitiation complex by recruiting RNA polymerase II to the core promoter. RNA polymerase II and the GTFs are referred to as the basal transcriptional machinery and are sufficient to mediate basal transcription (Naar, et al. 2001). Enhancer and silencer elements contain recognition sites for a variety of sequence-specific DNA-binding proteins, so called transcription factors. These proteins play a crucial role in regulating transcription in response to various signals (Levine, et al. 2003).

Most transcription factors interact with the proximal promoter region (about -40 to -250 relative to the +1 start site) (Kadonaga 2002). However, they can also interact with enhancers or silencer elements located several kilobases from the promoter. Transcription factors can either increase (activators) or decrease (repressors) the basal level of transcription. In general, transcription factors contain two domains, a DNA-binding domain involved in interactions with DNA and a domain involved in regulating transcription. There are exceptions to this rule, such as transcription factors that contain both activation and repression domains, as well as transcription factors that lack DNA-binding domains and instead are recruited to promoters through interactions with other proteins (Sharrocks 2000). Transcription factors often
interact with other transcription factors and/or coactivators/corepressors and work in a coordinated manner to regulate transcription.

It has been demonstrated that activators can affect several steps in the transcriptional process, including chromatin remodeling, preinitiation complex formation, recruitment of the holoenzyme, promoter clearance and transcriptional elongation (Roberts 2000). The mechanisms involved in transcriptional activation, especially the role of coactivators, will be discussed later. To date, three general mechanisms by which transcription can be repressed have been described. Firstly, a repressor can prevent the association of a transcriptional activator with the promoter. Secondly, a repressor can suppress the function of a DNA-bound transcriptional activator. Lastly, a repressor can have negative effects on the generation of an active transcriptional complex (Roberts 2000). It is common that repressors have overlapping functions. For example, Rb can function as a repressor in two different ways, either by inhibiting the activation domain of E2F or by recruiting HDAC to the promoter (Brehm, et al. 1998; Brehm, et al. 1999).

The levels and activities of transcription factors have to be tightly controlled since they are major players in gene regulation. This can be achieved in many different ways, including different posttranslational modifications, regulated localization and effects on the synthesis or degradation of the transcription factor. Several regulatory mechanisms often work together in a coordinated manner to control gene expression (Figure 9) (Sharrocks 2000).

![Regulation of transcription factors through posttranslational modifications.](image)

A common regulatory mechanism, especially for rapid alterations in transcription factor activity, is protein phosphorylation and dephosphorylation. This modification can affect the function of transcription factors either posi-
tively or negatively by affecting cellular localization, protein stability, protein-protein interactions or DNA-binding (Whitmarsh, et al. 2000). In addition, the ubiquitin-proteasome pathway plays an important role in the regulation of a large number of transcription factors. Rapid degradation of transcription factors allows cells to quickly respond to environmental stimuli. Many transcription factors that are only required for a short time during the cell cycle are regulated by ubiquitin-dependent proteolysis. In addition, other ubiquitin-like modifications, such as sumoylation, are also involved in the regulation of transcription factors. Although sumoylation does not target proteins for degradation, it may change their properties and alter their accessibility for degradation (Desterro, et al. 2000).

Acetylation of specific lysine residues in nucleosome-associated histones correlates with active transcription. Recent studies show that histones are not the only proteins targeted by acetylation, but also nuclear import factors, α-tubulin and transcription factors are acetylated. The number of acetylated transcription factors, as well as the number of enzymes catalyzing these reactions, has increased tremendously in the last few years. Also, deacetylation of non-histone substrates has been described, indicating that these reactions are reversible (Kouzarides 2000).


Besides affecting DNA-binding, acetylation can regulate protein-protein interactions. One example is the dissociation of nuclear esterogen receptors from their coactivator ACTR upon acetylation of ACTR by p300/CBP (Chen, et al. 1999). Furthermore, it has been shown that the general transcription factor TFIIB acetylates itself and that this stabilizes the interaction between TFIIB and the transcription factor TFIIF (Choi, et al. 2003). These two examples suggest that acetylation can regulate protein-protein interactions both negatively and positively. Acetylation of a lysine residue within the nuclear localization sequence of HNF-4 is crucial for proper nuclear retention, since non-acetylated HNF-4 is transported out to the cytoplasm via the CRM1 pathway (Soutoglou, et al. 2000), suggesting that acetylation may be important also for protein localization.

Another function regulated by acetylation is protein stability. It has been observed that the half-life of E2F1 is increased in response to acetylation (Martinez-Balbas, et al. 2000). The same is true for p53 (Ito, et al. 2001). A
mechanism for the acetylation-dependent stabilization of Smad7 was sug-
gested by the finding that the acetylation occurred on the same lysine resi-
dues as those targeted by ubiquitylation, thereby protecting the protein from
ubiquitylation (Grönroos, et al. 2002). It was subsequently shown that the
transcription factor SREBP1a is stabilized by a similar mechanism following
acetylation (Giandomenico, et al. 2003). The opposite is true for hypoxia-
inducible factor 1 (HIF-1), since acetylation leads to proteasomal degrada-
tion of the transcription factor (Jeong, et al. 2002).

Despite the increasing number of acetylated non-histone proteins, only a
few of these proteins are known to be deacetylated. So far, active deacetyla-
tion has been reported for E2F1, p53, YY1, α-tubulin and Smad7 (Luo, et al.

TGF-β and transcription

The TGF-β-dependent regulation of processes such as proliferation, differen-
tiation and apoptosis is achieved by controlling gene expression. Thus, the
transcriptionally active Smad proteins are of critical importance for the TGF-
β pathway (Massagué, et al. 2005). The focus of TGF-β signaling in recent
years has been to understand the complexity of TGF-β-dependent gene regu-
lation and how TGF-β is able to activate and/or repress hundreds of different
target genes at the same time in the same cell.

Upon TGF-β stimulation, Smads translocate to the nucleus where they
regulate transcription of target genes either by directly binding to consensus
DNA sequences in the promotors of these genes or through interactions with
other transcription factors or cofactors (Miyazono, et al. 2000). Smad3 and
Smad4 bind to a specific DNA sequence (5’-AGAC-3’), termed SBE (Smad
binding element) (Dennler, et al. 1998; Zawel, et al. 1998). However, Smads
can also bind GC-rich regions (Ishida, et al. 2000). Furthermore, there must
be a certain degree of tolerance in the interaction between the MH1 domain
and DNA; since some TGF-β-responsive regions lack both SBEs and GC-
rich regions, but are still able to bind Smads. This has been reported for the
binding of the Smad3/4 complex to the TGF-β inhibitory element (TIE) in
the c-Myc promoter (Chen, et al. 2002).

The crystal structure of the MH1 domain of Smad3 bound to the SBE
shows that a β-hairpin loop is responsible for the binding to DNA. The β-
hairpin sequence is conserved in all R-Smads and in Smad4, indicating that
DNA binding alone is unable provide target gene selectivity (Shi, et al.
1998). Despite the high degree of similarity between Smad2 and Smad3,
Smad2 is unable to bind DNA, due to a 30 amino acid insertion before the β-
hairpin loop (Figure 10) (Shi, et al. 1998). The 30 amino acid insert in
Smad2 is encoded by exon three, and a splice variant named Smad2ΔE3
lacking exon three has been described. This splice variant is not as abundant as Smad2, with the ratio in different tissues ranging from 3:1 to 10:1 (Dunn, et al. 2005). Smad2-null mice display an early embryonic lethal phenotype (Waldrip, et al. 1998; Heyer, et al. 1999), while mice engineered to express Smad2ΔE3 from the Smad2 locus are viable and fertile, indicating that full-length Smad2 is not required for viability (Dunn, et al. 2005). Similar to Smad3, the short splice variant of Smad2 is able to bind DNA (Yagi, et al. 1999). Moreover, Smad3 and Smad2ΔE3 form oligomeric complexes under uninduced conditions, whereas Smad2 is essentially monomeric under these conditions (Jayaraman, et al. 2000).

**Figure 10.** Exon three in Smad2 is located close to the β-hairpin loop and prevents DNA binding. The sequence of exon three is indicated in bold.

The stoichiometry of Smad complexes on promoters is still controversial. It has been proposed that heterotrimers consisting of two R-Smads and one Smad4 molecule interact with target promoters, whereas other studies favor heterodimers consisting of one R-Smad and one Smad4 molecule (Massagué, et al. 2005). Furthermore, studies of endogenous Smad complexes suggest an even more complex scenario, involving both heterodimers and heterotrimers depending on the target gene as well as the other factors present in the complex (Inman, et al. 2002).

Smads must interact with other DNA-binding proteins to achieve high affinity and selectivity for target genes. Smads bind with low affinity to DNA and the DNA sequence that they recognize occurs rather frequently in the genome. Thus, high-affinity binding of the Smads to their recognition motif would result in a rather general and unspecific expression of target genes (Feng, et al. 2005). FAST (forkhead activin signal transducer), is the most studied transcriptional partner of the Smad proteins (Chen, et al. 1996; Chen, et al. 1997; Labbé, et al. 1998; Zhou, et al. 1998; Liu, et al. 1999). A complex consisting of Smad2, Smad4 and FAST has been shown to activate the Mix-2 and goosecoid genes upon TGF-β and activin stimulation (Chen, et al. 1996).
FAST binds to DNA but is unable to activate transcription on its own and therefore needs to recruit the Smad2/4 complex. Interestingly, it has been demonstrated that Smad3 also binds FAST, but this complex inhibits activation of the goosecoid promoter (Attisano, et al. 2001).

Most of the known transcription-related interaction partners for Smads can function independently of Smads. Rather than being absolutely necessary for the transcriptional activity, Smads confer an additional level of regulation to these proteins by recruiting either coactivators or corepressors (Attisano, et al. 2000). This cooperation between Smads and other transcription factors also gives rise to crosstalk between different signaling pathways. One example of this is the cooperative action of Smads and the transcription factor ATF2. ATF2 is activated by the p38 pathway and inhibition of ATF2 or p38 blocks the ability of Smads to regulate ATF2-dependent promoter-reporter genes, indicating crosstalk between the Smad and p38 pathways (Hanafusa, et al. 1999; Sano, et al. 1999). Another mechanism that Smads use to activate transcription is relief of repression, exemplified by the ability of Smad1 to prevent binding of the Hoxc-8 repressor to DNA (Shi, et al. 1999).

Some genes are preferentially activated by either Smad2 or Smad3. One explanation for this could be that Smad2 and Smad3 have different affinities for the cofactors important for the activation of that particular gene. For example, regulation of c-Myc, Id1 and p21 prefers Smad3 over Smad2, which could be explained by the fact that Smad3 interacts better with the cofactors E2F4/5, ATF3 and FoxO (Chen, et al. 2002; Kang, et al. 2003; Seoane, et al. 2004).

Smads can activate transcription by recruiting transcriptional coactivators such as p300 and CBP (Massagué, et al. 2000). p300 and CBP can activate transcription by connecting transcription factors to the basal transcriptional machinery, as well as by acetylating histones and transcription factors (Chan, et al. 2001). Acetylated histones are associated with an open chromatin structure and therefore promote active transcription (Sterner, et al. 2000; Roth, et al. 2001). Moreover, Smad3 has also been shown to interact with the acetyltransferase P/CAF, resulting in an increased transcriptional activity (Itoh, et al. 2000). Interestingly, it has been demonstrated that the levels of p300 are increased upon TGF-β stimulation of normal fibroblasts, suggesting an additional mechanism by which TGF-β can regulate transcription (Bhattacharyya, et al. 2005).

Smads are not only able to activate transcription, they can also repress transcription. One way for Smads to repress transcription is by recruiting corepressors such as TGIF and Ski/SnoN to promoters (Akiyoshi, et al. 1999; Luo, et al. 1999; Stroschein, et al. 1999; Sun, et al. 1999; Wotton, et al. 1999). These corepressors will recruit histone deacetylases to the promoter, resulting in transcriptional repression. Moreover, it has been demon-
Stratford that Smad5 can recruit the methyltransferase Suv39h to the muscle creatine kinase promoter, resulting in transcriptional repression. Suv39h proteins methylate specific residues in histones, resulting in transcriptional repression of target genes (Frontelo, et al. 2004).

Figure 11. Schematic illustration of how TGF-β can regulate transcription.

If a TGF-β regulated gene will be expressed or not is regulated by the competition between coactivators and corepressors for binding to Smads (Massagué, et al. 2005) (Figure 11). It has been shown that Smad3, Smad6 and Smad7 can interact with HDACs (Ichijo, et al. 2005; Kang, et al. 2005; Simonsson, et al. 2005), and a Smad3-HDAC complex has been reported to repress the osteocalcin promoter (Kang, et al. 2005). An additional mechanism for Smads to repress transcription is to inhibit the action of transcriptional activators. For example, the interaction between Smad3 and MyoD prevents the formation of a transcriptionally active complex (Liu, et al. 2001).
Histone acetyltransferases (HATs)

Acetyltransferases are enzymes that transfer the acetyl group from acetyl-coenzyme A to the α-amino group of amino-terminal residues or to the ε-amino group of certain lysine residues. Among all posttranslational modifications, acetylation is the most frequent modification, at least for eukaryotic proteins (Polevoda, et al. 2002). Lysine residues in the N-terminal tails of histones are acetylated by a specific group of acetyltransferases, so called histone acetyltransferases (HATs). HATs can be divided into two general classes based on their cellular distribution and function. The type B HATs are located in the cytoplasm, and are believed to have a housekeeping role in cells. These proteins acetylate newly synthesized free histones in the cytoplasm for transport into the nucleus. The type A HATs are nuclear and catalyze transcription-related acetylation events (Sterner, et al. 2000; Roth, et al. 2001). The first type A HAT to be discovered was GCN5, which was already known to be a transcriptional coactivator (Brownell, et al. 1996). Subsequently, numerous coactivators/adaptors were found to possess intrinsic HAT activity. The HATs can be subdivided into five families according to their structural similarities. These include the GNAT, MYST, p300/CBP, the general transcription factor HATs and the nuclear hormone-related HATs (Carrozza, et al. 2003). Two of the best characterized families are the GNAT family, to which GCN5 and P/CAF belong, and the p300/CBP family (Kouzarides 2000; Martinez-Balbas, et al. 2000). The GNAT family has four conserved structural motifs, while the MYST family, to which MOZ, SAS2 and Tip60 belong, only shows similarity within the acetyl-CoA binding domain with the GNAT family. Also p300/CBP contains this acetyl-CoA binding domain. Except for the acetyl-CoA binding motif, the different families share little structural similarity (Roth, et al. 2001). Two of the most widely studied HATs associated with transcriptional regulation are p300 and CBP. These proteins share extensive amino acid similarity and have overlapping functions and are, therefore, often referred to as p300/CBP. p300/CBP are transcriptional coactivators and play important roles in a wide variety of cellular processes, including differentiation, apoptosis and cell cycle control (Giordano, et al. 1999). Mutations in p300 and CBP have been found in certain cancers, as well as some other human diseases, and both proteins are classified as tumor suppressors (Giles, et al. 1998; Goodman, et al. 2000; Kalkhoven 2004). p300/CBP function as coactivators in several different ways. They can act as protein bridges, connecting transcription factors with
the basic transcriptional machinery. Another key function of p300/CBP is to assist in the formation of transcriptional complexes by working as scaffold proteins. The HAT activity of p300/CBP is important for histone acetylation, as well as for the acetylation of non-histone substrates (Chan, et al. 2001). p300/CBP are large proteins of about 300 kDa and contain several conserved regions, including a bromodomain, three cysteine/histidine-rich domains, a KIX domain and an ADA2-homology domain. The cysteine/histidine-rich domains and the KIX domain are important for many of the protein-protein interactions that p300/CBP are involved in (Chan, et al. 2001; Blobel 2002).

The activity of HATs is regulated by posttranslational modifications, interactions with other proteins, as well as through regulated expression of the corresponding genes (Blobel 2002; Legube, et al. 2003). For example, phosphorylation of CBP by CDK2 stimulates its HAT activity (Ait-Si-Ali, et al. 1998). Several viral and cellular proteins can modulate the activity of specific HATs. Some examples are E1A, E1B, viral interferon factor, twist and Mdm2 (Chen, et al. 2001; Blobel 2002).
Histone deacetylases (HDACs)

Acetylation is a reversible process and the removal of the acetyl group is catalyzed by histone deacetylases (HDACs). Eighteen HDACs have been described and they can be divided into three different classes based on their homology to yeast HDAC proteins. The class I HDACs includes HDAC1, -2, -3, -8 and -11 and they are homologous to yeast RPD3 (HDAC11 also has similarities to class II HDACs) (Gao, et al. 2002; Verdin, et al. 2003). This group contains a well-conserved catalytic domain that encompasses almost two thirds of the proteins. The class I HDACs are mainly nuclear, except for HDAC3 which shuttle between the nucleus and the cytoplasm (Khochbin, et al. 2001; de Ruijter, et al. 2003). HDAC1 and HDAC2 are members of two multiprotein complexes, the Sin3/HDAC and NuRD/Mi2/NRD complexes, while HDAC3 associates with the N-CoR and SMRT corepressor complexes (Knoepfler, et al. 1999; Wen, et al. 2000). All class I HDACs are expressed in most tissues and cell lines (Fischle, et al. 2001). The class II HDACs are homologous to yeast HDA1 and includes HDAC4, -5, -6, -7, -9 and -10. The class II HDACs are subdivided into two subclasses, Iia (HDAC4, -5, -7 and -9) and Iib (HDAC6 and -10) (Verdin, et al. 2003). The class II HDACs are much larger than class I HDACs and HDAC6 and -10 contain two catalytic domains (Guardiola, et al. 2002). Class II HDACs are regulated by nuclear-cytoplasmic shuttling, which is believed to be of major importance for their function. In contrast to class I HDACs, class II HDACs exhibit tissue-specific expression, suggesting that they could be involved in cellular differentiation and/or development (Fischle, et al. 2001).

The function of HDACs can be regulated in several ways, such as subcellular localization, the formation of multisubunit complexes and posttranslational modifications, including phosphorylation and sumoylation (Pflum, et al. 2001; David, et al. 2002; Galasinski, et al. 2002; Kirsh, et al. 2002; Yang, et al. 2003). Members of the third class of HDACs are homologs of yeast SIR2 and is dependent on NAD⁺ for their enzymatic activity. The class III HDACs are most likely involved in functions not shared with the other HDACs (Khochbin, et al. 2001).
Present investigation

Aim
The focus of my work has been to study the role of protein acetylation in TGF-β signaling. My specific aims have been:

I To investigate whether the acetylation of Smad7 is a reversible process and to assess the importance of this process.

II To determine if R-Smads and/or Smad4 are acetylated, and if so, clarify the functional consequence(s) of this modification.

III To address the possibility that certain Smad proteins have intrinsic acetyltransferase activity.

Paper I
The balance between acetylation and deacetylation controls Smad7 stability

It was previously shown that Smad7 is acetylated, resulting in its stabilization. The acetylation was mapped to two specific lysine residues. Interestingly, the same lysine residues were targeted for ubiquitylation, explaining the increased stability of Smad7 in response to acetylation (Grönroos, et al. 2002). These findings suggested a model in which the acetylation/ubiquitylation of specific lysine residues functions as a switch to regulate protein stability. However, this model required that the acetylation of Smad7 was reversible. We therefore wanted to determine if Smad7 was deacetylated and identify the deacetylases involved. This lead to the following question, if deacetylation occurs, will this result in the degradation of Smad7?
In Paper I, we demonstrate that Smad7 interacts with both class I and class II HDACs and that Smad7 is a substrate for specific HDACs. An increase in the acetylation of Smad7 was observed when cells were treated with TSA, a general HDAC inhibitor, indicating that Smad7 is actively deacetylated by HDACs. Moreover, we could show that specific HDACs deacetylated Smad7 in vivo. These findings correlated well with the results of our interaction studies, where the class I HDACs HDAC1 and -3 interacted well with Smad7, while only a weak interaction was detected between Smad7 and HDAC2. The class II HDACs, HDAC5 and -6, displayed weaker interactions with Smad7 than the class I HDACs, and no interaction between Smad7 and HDAC4 could be detected.

We focused our work on HDAC1, since the strongest interaction and deacetylation was observed when Smad7 was co-expressed with HDAC1. The interaction between Smad7 and HDAC1 is most likely direct, since we were able to detect an interaction in GST pull-down assays with GST-HDAC1 and Smad7 expressed in 293T cells. Surprisingly, GST-Smad7 did not interact with HDAC1, which could suggest that Smad7 needs to be modified in order to interact with HDAC1. We mapped the interaction domains in both Smad7 and HDAC1 and found that the MH2 domain of Smad7 was important for its interaction with HDAC1. The catalytic region in HDAC1 was important for its interaction with Smad7. It was demonstrated earlier that the acetylation of Smad7 was decreased upon TGF-β treatment. This could depend on several factors, such as enhanced nuclear export of Smad7 or an increased deacetylation of Smad7 by HDAC1. We were unable to detect any TGF-β-dependency in the interaction between Smad7 and HDAC1. This could be due to the fact that both proteins were overexpressed in our interaction studies, which most likely resulted in an unnatural subcellular distribution of the proteins.

It has been suggested that acetylation protects proteins such as Smad7, SREBP and e-Myc from ubiquitylation and degradation. We could strengthen this hypothesis by showing that the deacetylation of Smad7 negatively affected its stability. We observed an increase in the ubiquitylation of Smad7 when it was coexpressed with HDAC1. Consequently, we observed a decrease in the levels of Smad7 when it was cotransfected with HDAC1. The decrease in Smad7 levels in response to HDAC1 expression could be overcome by treating the cells with TSA. The data presented support a model in which the balance between the acetylation and deacetylation of Smad7 determines its stability (Figure 12).
Figure 12. Acetylation - a dynamic regulator of protein stability. Adapted from Simonsson et al., 2005.

Paper II

The DNA binding activities of Smad2 and Smad3 are regulated by coactivator-mediated acetylation

In order to further study the importance of protein acetylation in TGF-β signaling, we investigated if any of the R-Smads or Smad4 were acetylated in vivo. To analyze this, the different Smad proteins were expressed in 293T cells in the absence or presence of the acetyltransferases p300, CBP or P/CAF. The Smads were immunoprecipitated and the acetylation was determined with a general acetyl-lysine antibody. We found that Smad2, Smad2ΔE3 and Smad3 were acetylated in vivo, whereas we were unable to detect any acetylation of Smad4.

The most prominent acetylation was observed when Smad2 was expressed in the presence of p300. The acetylation of Smad3 was always lower than that of Smad2. Moreover, Smad2 and Smad2ΔE3 were acetylated by P/CAF, while we were unable to detect any P/CAF-dependent acetylation of Smad3. These results differ from our previous work with Smad7, since Smad7 was only acetylated by p300. We observed an increase in the acetylation of the transfected R-Smads in response to constitutively active ALK5 receptor, as well as an increase in the acetylation of endogenous Smad2 when cells were treated with TGF-β. These results suggested that the acetylation of R-Smads is stimulated by TGF-β. The latter results differ from those obtained with Smad7, since TGF-β stimulation decreased the acetylation of Smad7.
We wanted to identify the lysine residues acetylated in the R-Smads. To address this issue, we made point-mutations of lysine residues in Smad2 and tested the mutant proteins in *in vivo* acetylation assays. We could demonstrate that the major acetylation site in Smad2, Smad2ΔE3 and Smad3 is lysine 19. Interestingly, mutation of an adjacent lysine residue (lysine 20) did not affect the acetylation of Smad2.

We focused on Smad2 and Smad2ΔE3 since these proteins were acetylated more efficiently than Smad3. In order to study the acetylation of lysine 19, we raised an acetylation-specific antibody against this residue in Smad2. Using this antibody, we were able to detect acetylation of endogenous Smad2 in HaCaT cells. We observed an increase in the acetylation of Smad2 when cells were treated with TGF-β, and a further increase in acetylation when cells were treated with the HDAC inhibitor TSA. The latter result suggested that the acetylation of Smad2 could be reversible. No acetylation of endogenous Smad2 was observed when HaCaT cells were treated with anacardic acid, an inhibitor of p300 and P/CAF. These results were confirmed when U2OS cells were treated with short hairpin RNA directed against p300 and P/CAF. Inactivation of both P/CAF and p300 resulted in a reduction in the acetylation of endogenous Smad2. No significant decrease in the acetylation of Smad2 was observed when short hairpin RNA against CBP was used, suggesting that p300 and P/CAF are of major importance for the acetylation of Smad2.

Acetylation has attracted a lot of interest in recent years since it regulates a wide variety of protein functions. In Paper I, we demonstrated that the acetylation of Smad7 is important for its stability and that the acetylation is reversible. We were, therefore, interested to know if this was true also for Smad2 and Smad2ΔE3. The conclusion of our studies was that acetylation does not influence the stability of Smad2, nor does it influence the phosphorylation status of Smad2.

Since the phosphorylation of Smad2 was unaffected by acetylation, we did not expect to see any effects on its interactions with common interaction partners such as Smad4, Smad3, FAST or the acetyltransferases CBP, p300 and P/CAF. Furthermore, most interactions between Smad2 and other proteins are dependent on the MH2 domain and acetylation occurs in the MH1 domain. Coimmunoprecipitation assays were performed to analyze if the interactions with Smad4, Smad3, FAST or the acetyltransferases were influenced by acetylation of lysine 19. As expected, we were unable to detect any significant differences between wildtype Smad2 and the K19R mutant of Smad2 in these experiments.

In order to investigate the functional importance of the acetylation of Smad2, we performed transcriptional promoter-reporter assays. We observed a significant increase in transcriptional activity of wildtype Smad2ΔE3 on the 12xCAGA promoter in response to p300 and P/CAF. In contrast, no significant increase was observed for the K19R mutant. These findings sug-
gested that the acetylation of Smad2ΔE3 could be important for its transcriptional activity. Interestingly, we observed no significant difference in transcriptional activity between wildtype Smad2ΔE3 and the K19R mutant when these proteins were fused to the DNA-binding domain of Gal4 and analyzed on a Gal4-responsive promoter. This suggested that the acetylation of Smad2ΔE3 could influence its DNA binding activity. To test this hypothesis, we performed electromobility shift assays (EMSA}s). Wildtype and the K19R mutant of Smad2ΔE3 were transfected in Cos cells with or without cotransfection of p300 or P/CAF. Whole cell lysates from the transfected cells were used in EMSAs. Coexpression with p300 and P/CAF enhanced the DNA binding activity of wildtype Smad2ΔE3, whereas no binding was detected when the K19R mutant was used. We also performed EMSAs with \textit{in vitro} translated proteins and recombinant GST–Smad2, all supporting the notion that acetylation of lysine 19 in Smad2ΔE3 enhances its DNA binding. This hypothesis was strengthened by chromatin immunoprecipitation (ChIP) assays, which were performed with both transfected material and endogenous Smad2. The PAI-1 and p21 promoters were studied in the ChIP assays. When transfected material was used, the association of wildtype Smad2ΔE3 with the 12xCAGA or PAI-1 promoters was increased in the presence of p300, whereas no binding was observed for the K19R mutant. When studying endogenous Smad2, we were able to detect an increased binding of acetylated Smad2 to both the PAI-1 and p21 promoters in response to TGF-\(\beta\) stimulation. In addition, the ChIP assays demonstrated that p300 and P/CAF were recruited to these promoters with the same kinetics as Smad2. Taken together, our data support the hypothesis that acetylation of lysine 19 in Smad2ΔE3 promotes its DNA binding.

\textbf{Figure 13.} Acetylation may influence the structure of Smad2ΔE3, thereby promoting DNA binding.
We also tried to determine how the acetylation of lysine 19 in Smad2ΔE3 could enhance its DNA-binding activity. We speculated that acetylation may induce a conformational change that could expose the DNA binding domain in the MH1 domain, thereby making it more accessible to DNA. To test this hypothesis, we performed protease-protection assays. Wildtype Smad2ΔE3 and the K19R and K19Q mutants were in vitro translated and treated with thrombin. We observed different digestion patterns for the different proteins. The K19Q mutant (mimicking acetylated Smad2) was susceptible to thrombin digestion, whereas the wildtype protein and the K19R mutant (mimicking non-acetylated Smad2) were resistant to thrombin cleavage. These results suggest that acetylation of lysine 19 in Smad2ΔE3 could influence the structure in a way that favors DNA binding (Figure 13). Similar experiments were performed with Smad2FL, giving similar results. In conclusion, we have demonstrated that the R-Smads are substrates of p300, CBP and P/CAF. The acetylation is TGF-β-dependent and lysine 19 is the major acetylation site in Smad2, Smad2ΔE3 and Smad3. Most likely, acetylation results in a conformational change which positively affects the DNA binding activity of Smad2ΔE3. The increased association of Smad2ΔE3 with target promoters following acetylation results in an increased transcriptional activity.

**Paper III**

*The acetyltransferase activity of Smad2 regulates its transcriptional activity*

In paper II, we showed that Smad2FL, Smad2ΔE3 and Smad3 are acetylated by p300, CBP and P/CAF. During these studies, we also observed that purified Smad2FL became acetylated in in vitro acetylation assays without any addition of p300, indicating that Smad2 may have intrinsic acetyltransferase activity. Further investigations showed that Smad2FL, Smad2ΔE3 and Smad7 all possessed intrinsic acetyltransferase activity in vitro. Limited or no acetyltransferase activity was observed for Smad3 and Smad4. The acetyltransferase activity was not only observed with bacterially expressed Smad2, but could also be observed with Flag-tagged Smad2 immunoprecipitated from 293T cells. The acetyltransferase activity of Smad2FL was attenuated when three different well-established acetyltransferase inhibitors were used. Moreover, no acetylation was observed when Smad2 was heat-inactivated at 65 °C or when the acetylation reaction was performed at 4 °C. These results suggested that the modification was not a result of nonenzymatic acetylation. Deletion mutants of GST-Smad2 indicated that both the acetyltransferase activity and the autoacetylated lysine residue(s) were localized to the N-terminus of Smad2. In paper II, we found that lysine 19 in
Smad2 and Smad2ΔE3 was the major acetylation site targeted by p300/CBP and P/CAF. In paper III, we demonstrate that the same lysine residue is the major autoacetylation site in Smad2FL and Smad2ΔE3.

Most protein acetyltransferases are able to acetylate histones or other proteins. However, we have not yet been able to show any Smad2-dependent acetylation of histones or other proteins in pure in vitro acetylation assays. However, these results do not exclude the possibility that Smad2 is able to do this in vivo or in the context of chromatin. Despite a high degree of similarity, Smad2 and Smad3 differ in their MH1 domains. Smad2 contains two specific amino acid stretches which Smad3 lacks. One of these regions is called GAG and is Gly-rich and contains the sequence GxGxxG, a motif often found in CoA-binding proteins. This motif is similar to the CoA-binding motif found within motif A of the GCN5 family of acetyltransferases. Interestingly, we found that the autoacetylation of Smad2 was lost when the GAG region was deleted, while Smad3 gained acetyltransferase activity when the GAG region was introduced in Smad3. To further address the importance of the GAG domain, the three Gly residues in the potential CoA-binding motif were mutated to valines. As a positive control, the corresponding amino acids were mutated in P/CAF. As expected, these mutations in Smad2 and P/CAF blocked their autoacetylation. Moreover, we could demonstrate in CoA binding assays that the interaction between Smad2 and CoA was lost in the Gly mutant. These results suggested that the GAG region in Smad2 is important for acetyl-CoA binding and that this motif is important for the AT activity of Smad2. Many acetyltransferases contain zinc fingers in close proximity to their AT domains and for some it has been shown to be important for their AT activity. Smad2 also contains a zinc finger in its MH1 domain and we found that mutations of this domain disrupted its acetyltransferase activity, further supporting the notion that the AT activity of Smad2 is a result of an enzymatic activity.

To strengthen our hypothesis that the MH1 domain of Smad2 contains intrinsic AT activity and to prove that this activity is not a result of a copurifying bacterial acetyltransferase, we performed size-exclusion chromatography. Wildtype GST-Smad2FL and the acetylation-deficient 3GV mutant eluted in the same fraction, but only the wildtype protein contained AT activity. These results demonstrate that the AT activity is intrinsic to the purified Smad2 protein.

To address the functional consequence of the autoacetylation of Smad2, we performed promoter-reporter gene assays with both Smad2FL and Smad2ΔE3. Both Smad2FL and Smad2ΔE3 function as transcription factors, but only Smad2ΔE3 is able to directly bind DNA. In the transcriptional assays, wildtype Smad2 was compared to the corresponding 3GV mutant. Both in the case of Smad2FL and Smad2ΔE3, we observed a reduction in transcriptional activity in the AT-deficient mutant. This suggested that the AT activity of Smad2 is important for its transcriptional activity. In line with our
observations in paper II, we could demonstrate that there was no difference in transcriptional activity between wildtype Smad2ΔE3 and the 3GV mutant when fused to the DNA binding domain of Gal4. These results indicate that the AT activity may influence the DNA binding activity of Smad2ΔE3. However, we observed a difference in transcriptional activity between wildtype Smad2FL and the 3GV mutant when these proteins were fused to the DNA binding domain of Gal4, suggesting that the AT activity is not influencing the DNA binding activity of Smad2FL.

To determine if the AT activity of Smad2ΔE3 affected its DNA binding activity in vitro, the DNA binding activities of wildtype Smad2ΔE3 and the 3GV mutant were compared in EMSAs following autoacetylation. The results indicated that the AT activity of Smad2ΔE3 is important for its DNA binding activity. These results were strengthened by ChIP assays in HepG2 cells, where wildtype Smad2ΔE3, but not the 3GV mutant, was associated with the PAI-1 promoter after TGF-β stimulation.

In paper III, we demonstrate that Smad2FL, Smad2ΔE3 and Smad7 possess intrinsic acetyltransferase activity in vitro. Moreover, we show that the GAG region in Smad2 is important for its binding to acetyl-CoA and map the major autoacetylation site to lysine 19. We also demonstrate that the AT activities of Smad2FL and Smad2ΔE3 enhance their transcriptional activities. In the case of Smad2ΔE3, autoacetylation enhances its DNA binding activity. We were unable to observe any Smad2-dependent acetylation of histones or other proteins in vitro, but this does not exclude the possibility that Smad2 may have other substrates in vivo.
Future perspectives

TGF-β1 was discovered more than three decades ago. Despite the time that has past since its discovery, there are still many discoveries that have to be made in order to understand this fascinating factor. The importance of the TGF-β signaling pathway, both for cells and for the whole organism, is demonstrated by the fact that the pathway is well conserved in all organisms and that malfunction results in pathological conditions.

The focus of my work has been to further understand the intracellular signaling of Smads and how these proteins are regulated by posttranslational modifications, especially acetylation.

Acetylation was originally shown to occur on histones and correlate with active transcription. Later on, it was demonstrated that also other proteins could be acetylated. Acetylation has been shown to influence a wide variety of functions, including protein-protein interactions, protein-DNA interactions, protein localization, as well as protein stability (Kouzarides 2000). Acetylation is a reversible process and the acetyl group can be removed by histone deacetylases (HDACs).

When I began my thesis work, it was already established that Smad7 was acetylated by p300 and the major acetylation sites had been mapped to lysines 64 and 70 (Grönroos et al. 2002). In that study, the authors also showed that the acetylation of Smad7 protected the protein from ubiquitin-dependent degradation. This was the first time a Smad protein was found to be acetylated. Showing that acetylation and ubiquitylation can occur on the same lysine residues and that acetylation protects the protein from proteosomal degradation opened up a new way of thinking about how protein stability could be regulated. If this theory was correct, it indicated that the acetylation of Smad7 should be a reversible process.

The first part of my thesis focused on the reversibility of the acetylation of Smad7. In Paper I, we demonstrated that Smad7 interacts with both class I and class II HDACs. Furthermore, we showed that Smad7 is deacetylated, both in vivo and in vitro. Finally, we demonstrated that the deacetylation of Smad7 decreased its stability. These results support the hypothesis made by Grönroos et al., that acetylation and ubiquitylation of the same lysine residue in a protein is a regulatory mechanism. These data are supported by publications from other groups obtaining similar data for other proteins, including p53, E2F and SREBP (Martinez-Balbas, et al. 2000; Ito, et al. 2001; Giandomenico, et al. 2003). Taken together, these results suggest that the
acetylation and deacetylation of proteins is a general mechanism to regulate protein stability. Further support for our observations is provided by the recent finding that Smad7 can be deacetylated by a class III HDAC, resulting in the ubiquitylation and destabilization of Smad7 (Kume, et al. 2007). The deacetylation of Smad7 in mesangial cells reduced apoptosis, suggesting that up-regulation of the class III HDAC could be a potential therapeutic strategy in TGF-β-related kidney disease.

It is of great interest to know how the stability of Smad7 is regulated, since Smad7 antagonizes TGF-β signaling. TGF-β is known to promote the progression of some cancers at later stages. It would therefore be of interest to inhibit TGF-β function in these cancers. One possibility could be to use HDAC inhibitors to stabilize Smad7. A number of HDAC inhibitors are already used in clinical trials for cancer treatment and they are mainly thought to work on the transcriptional level. It would be interesting to use these compounds on tumors in which TGF-β promotes cancer progression to determine whether they will have any effect or not.

In Paper II, we demonstrated that R-Smads are acetylated, while no acetylation was observed for Smad4. We mapped the major acetylation site in Smad2FL, Smad2ΔE3 and Smad3 and show that the acetylation of this residue was TGF-β-dependent. Acetylation of Smad2 resulted in an increased transcriptional activity, both for Smad2FL and Smad2ΔE3. Since Smad2FL is unable to bind DNA by itself, we used Gal4-Smad2FL constructs that bind to promoters containing Gal4 binding sites. With this experimental set-up, we observed an increased transcriptional activity when Smad2FL was expressed together with p300 and P/CAF and this effect was not observed with the K19R mutant. These results suggested that the acetylation of Smad2FL promoted the formation of a transcriptionally active complex. Of course, it would be of great interest to further study the mechanisms involved in these processes. Both in vitro and in vivo DNA binding assays with Smad2ΔE3 suggested that acetylation promoted its DNA binding activity and, consequently, enhanced its transcriptional activity. Protease-protection assays with both Smad2FL and Smad2ΔE3 indicated that acetylation induces a structural change that opens up the structure of both proteins, making new surfaces accessible for interactions. Further studies are needed to confirm that structural changes occur in response to the acetylation of Smad2.

The results obtained in Paper II opens up a new way of thinking about TGF-β signaling. The old dogma, in which phosphorylation is central for the activation of Smads, is perhaps only one part of the truth. Perhaps the phosphorylation of R-Smads is only needed for their initial activation and transport to the nucleus. Once in the nucleus, Smads need to be activated by acetylation in order to be transcriptionally active. This is supported by the observation that acetylated GST-Smad2ΔE3, as well as the mutant mimicking acetylated Smad2, bound DNA in EMSAs, despite the fact that they were not phosphorylated. The acetylation of R-Smads provides a new way to
regulate TGF-β signaling and it would be interesting to know if R-Smads are deacetylated. Preliminary results with the general HDAC inhibitor TSA indicate that this is the case.

During the work with Paper II, we made the interesting observation that recombinant GST-Smad2 and -Smad7 became acetylated \textit{in vitro} without the addition of any acetyltransferases, suggesting that both proteins have intrinsic enzymatic activity. Moreover, we could show that the MH1 domain was important for the activity and that lysine 19 was a major target for the acetyltransferase activity of Smad2. We further demonstrated that a triple mutation in a Smad2-specific region (GAG) in close proximity to lysine 19 drastically reduced its enzymatic activity. This mutant displayed a reduced binding to CoA, suggesting that this could be the binding site for acetyl-CoA. Smad3 lacks this region and shows no enzymatic activity \textit{in vitro}, while Smad7 has several potential acetyl-CoA binding sites. By introducing the acetyl-CoA binding domain of Smad2 in Smad3, Smad3 gained enzymatic activity. We used different well-established acetyltransferase inhibitors to show that the AT activity of Smad2 was an enzymatic activity and not the result of non-specific binding of acetyl-CoA. Of course, a future challenge will be to find a substrate for Smad2 and we have speculated that histone tails may be targeted by the acetyltransferase activity of Smad2 \textit{in vivo}. Direct \textit{in vitro} acetylation assays using GST-Smad2 and recombinant histones have been tested without success. This could be due to several different problems associated with the assays. Our hypothesis is that the functions of Smad2 and Smad3 have diverged during evolution and that Smad3 works as a transcription factor by direct DNA binding, while Smad2 instead works as a coactivator by using its AT activity to acetylate histones and/or other proteins.
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