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THE 26S PROTEASOME SYSTEM IN THE SIGNALING PATHWAYS OF TGF-BETA SUPERFAMILY

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1. ABSTRACT

Recent studies of the Smad family proteins, which are the key signal transducers of the TGF-beta family ligands, have revealed the ability of Smads to interact with various components of the 26S proteasome system. Such interactions are now known to contribute to the regulation of Smad protein levels before and after Smad activation. Most importantly, such interactions are also shown to be an integral part of the signaling functions of Smads. Through a physical interaction with different ubiquitin E3 ligases (HECT family, SCF and APC complex), the TGF-beta/activin responsive Smad3 exhibits the novel ability to regulate the ubiquitination of several key regulators, such as the oncoprotein SnoN and the multi-domain docking protein HEF1. The proteasomal degradation of these two proteins links TGF-beta signaling to multiple signaling pathways involving SnoN and HEF1. Through the interaction with proteasome beta subunit HsN3 and the substrate marker protein ornithine decarboxylase antizyme (AZ), the BMP responsive Smad1 regulates the proteasomal targeting events that contribute to the degradation of Smad1 and its interacting proteins, one of which is SNIP1, a repressor of the transcriptional co-activator CBP/p300. Thus, the novel physical link between Smads and components in the 26S proteasome system allow the intracellular events triggered by the TGF-beta family ligands to connect with those induced by many other extracellular regulators, thereby forming an extremely complex signaling network to regulate a wide range of biological activities.

2. INTRODUCTION

The TGF-beta family cytokines exert broad biological activities, which range from early embryonic patterning, to tissue/organ morphogenesis, to the maintenance of the homeostasis of various systems in the body. In the past ten years, rapid progress has been made in dissecting the intracellular molecular events underlying the myriad biological activities of the TGF-beta superfamily (1-7). A major breakthrough was made when the key signal transducers of this family of ligands were identified (2-7). They are now known as the Smad family signal transducers, which have distinct structural and functional features which are not found in any other known signal transducers. How Smad proteins mediate such broad range regulatory roles of the TGF-beta family cytokines has been a main focus of study in the TGF-beta field within the past several years. To understand the functional mechanisms of this novel family of signal transducers, researchers in the field took two different approaches, a hypothesis-driven approach and a discovery-driven approach. The hypothesis-driven approach was initiated based upon the biochemical observations of the nuclear translocation of activated Smad proteins, which urged the investigation of Smads as receptor-activated transcriptional regulators. A second approach, the discovery approach, was based upon the observation that Smads form protein complexes with other unknown proteins, thus urging the "blind" search for Smad-interaction proteins using various means. The hypothesis-driven approach has now revealed the linear signaling events of Smad family proteins from the cell membrane to the nucleus. A relatively conserved cascade of events has been mapped for each TGF-beta family member. The linear pathway includes the initial activation of Smads by the membrane-associated receptors, the complex formation of heteromeric Smad complexes, the nuclear translocation of the Smad complexes, followed by the transcriptional regulation mediated by the interaction of Smad complexes with specific gene promoters. Molecular details of Smad-regulated transcription are not fully mapped, but have been shown to involve Smad binding to DNA, Smad interaction with other DNA-binding transcription factors, and the recruitment of transcriptional co-activators or co-repressors (2;4;5;7;8).

On the other hand, the discovery-driven approach has now revealed an amazingly complex picture of the signaling network of Smads. The first approach was initiated in 1996 as a collaborative project between Lechleider R. & Roberts A. and Wang T. & Donahoe P. groups. Inspired by the observation that the newly cloned BMP-responsive Smad1 protein, then called BSP1, can bind a large number of endogenous proteins in a GST pull down assay (9), a yeast two-hybrid screen was applied to examine the interactors of Smad1 (10). A similar screen was carried out for the TGF-beta responsive Smad3 (11). Strikingly, multiple proteins involved in the 26S proteasome system were identified from these two screens, as strong and specific interactors of these two Smads (10;12;13). Among them are: the two types of ubiquitin fusion proteins (Uba 52 and Uba 80) (14-18), the ornithine decarboxylase antizyme (19-21), the ubiquitin E3 ligase hItch (22;23), and a beta subunit of the 20S proteasome HsN3 (24-26). Besides these proteasome-related proteins, the rest of the Smad interactors include both cytoplasmic and nuclear proteins. To test a potential functional link between the activity of the 26S proteasome and the signaling pathways of BMPs and TGF-beta, a collaboration project was established to test the effects of proteasome inhibitors on BMP-induced dendritic growth of rat sympathetic neurons. Time- and dose-dependent inhibitory effects of proteasome inhibitors on BMP-induced dendritic growth were detected (13). Similar inhibitory effects were also detected in TGF-beta-induced gene responses (*B.-Y. Li et al.,* unpublished data). Based upon these observations, it was hypothesized that Smads are either targeted to the 26S proteasome for degradation, or play a role in regulating proteasomal degradation of other proteins, or both. Progress made in the past five years by many laboratories in the field has now confirmed and extended these initial observations. It is now well established that interactions between Smads and the components of the 26S proteasome play key roles in regulating protein levels of Smads before and after Smad activation, in down-regulation of TGF-beta type I receptor as a negative feedback mechanism, and in mediating both nuclear and cytoplasmic signaling events of Smads (10-12;27-38). Recent characterization of Smad3-dependent proteasomal degradation of the oncoproteins Ski and SnoN and the multi-domain cytoplasmic docking protein HEF1 or CasL suggests that Smad-dependent proteasomal degradation of nuclear and cytoplasmic

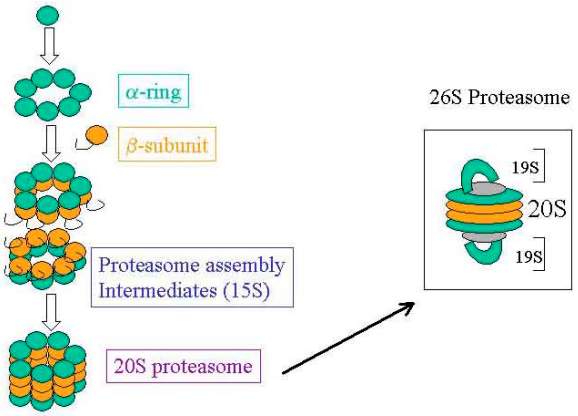

Figure 1. The assembly of the 26S proteasome. The alpha subunits (green color) first form a ring. The beta subunits (orange color) containing the prosequences (illustrated as a short tail) then assemble onto the alpha ring template to form a beta ring. The two half proteasomes then assemble into the 20S proteasome, which, upon interaction with the 19S complexes, form the 26S proteasome. The proseqences of the beta subunits are cleaved immediately before the assembly of the 20S proteasome.

regulators mediate large scale cross-talk between TGF-beta family ligands and those of others (11;34;35). Considering the existence of a large number of Smad interacting proteins and the intimate physical and functional link between Smads and the proteasome system, it is very likely that we are only seeing the "tip of the iceberg" of the abilities of Smads to mediate multi-level cross-talk in the cell. New data also suggest that Smad-regulated proteasomal degradation events could also underlie the well-observed transcriptional regulatory abilities of Smads (10). Thus, the many different functions of Smads in the cytoplasm and in the nucleus can be better understood by acknowledging the fundamental property of Smads as novel regulators of proteasomal degradation events.

This review outline known physical interactions between Smads and the 26S proteasome and their observed functional outcomes, and will then discuss the implications of this information for future studies of the regulation and functions of the Smad family signal transducers.

3. THE 26S PROTEASOME SYSTEM

The readers are referred to numerous excellent review articles in this field (39-43). Here only a brief introduction is given on relevant properties of the system.

3. 1. Structure and assembly of the 26S proteasome

The 26S proteasome is a large multi-subunit proteolytic complex first characterized in 1980s. It is responsible for the degradation of most of the cytosolic and nuclear proteins in eukaryotic cells. The 26S proteasome consists of three large sub-complexes: the 700 kDa catalytic core of four stacks of seven-subunit rings (two beta rings sandwiched by two alpha rings) and two 700 kDa regulatory complexes (also called the 19S complex, or PA700) at each end of the core cylinder. The proteolytic activities of the 20S proteasome are derived mostly, if not entirely, from two copies of three catalytic beta subunits residing within the two inner beta rings, named X, Y and Z subunits. In immune cells, IFNgamma triggers the switching of these three subunits into three distinct catalytic subunits LMP2, 7, and MECL1, which are specialized for antigen presentation (44;45).

The assembly of the 20S proteasome from 28 separately made subunits (two copies of seven α subunits and two copies of seven β subunits) involves complicated steps and many assembly intermediates (46-48). Figure 1 depicts some basic steps along the assembly process, which maybe subjected to different types of regulations, about which little is known except for two interesting aspects. First, a protein named UMP1, first found in yeast and now also found in mammals, is essential for normal assembly of the 20S proteasome (49;50). Lack of UMP1 in yeast led to the accumulation of assembly intermediates and the failure of complete assembly of the 20S proteasome. How UMP1 regulates the assembly is not fully understood, but it has been suggested to regulate the final assembly of the two half proteasomes (15S) into the mature proteasome (20S). Second, the prosequences of four out of seven different beta subunits are processed prior to the final assembly of the 20S proteasome. The prosequences serve at least two functions, to sequester the catalytic sites to prevent non-specific proteolysis and to serve as a chaperone sequence for correct incorporation of the beta subunits. Deletion of the proseqence of one beta subunit leads to severe assembly defects of the proteasome (51). The catalytic sites of the active beta subunits are exposed only upon processing of the N-terminal prosequence, an event occurs immediately prior to the full assembly of the 20S poteasome. Thus, the catalytic sites of the beta subunits are enclosed inside of the inner chamber of the cylinder immediately after they are exposed, ensuring that only proteins exposed to the inner chamber of the proteasome are subjected to degradation by these beta subunits. The size of the inner chamber is only 13 Å for the 20S proteasome of *Thermoplasma*, based upon crystallography (52). Thus, it is predicted that substrates need to be threaded inside of the chamber for degradation. The 19S complex, which is required for the 20S core to degrade protein substrates, is made of two distinct substructures: a base of six ATPases and a "cap" of more than 14 different non-ATPases. According to crystallographic findings of the yeast 26S proteasome, both ends of the 20S proteasome cylinder are totally sealed by the N-termini of the alpha subunits (53). How protein substrates enter the degradation chamber of the 20S proteasome is currently not clear, however, the ATPases are suggested to participate in unfolding of a protein substrate.

3. 2. The targeting of the protein substrates to the 26S proteasome

Two main pathways have been characterized for protein targeting to the 26S proteasome: the ubiquitin-dependent pathway and the antizyme-dependent pathway (for ornithine decarboxylase), as illustrated in Figure 2.

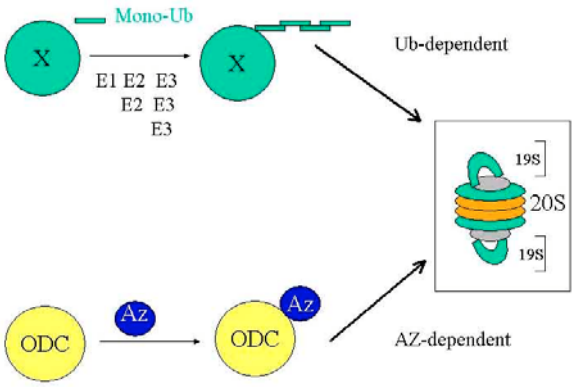

Figure 2. Two different substrate-targeting pathways for the 26S proteasome. The top illustrates the ubiquitin-dependent targeting pathway for most of the polyubiquitinated proteins, while the bottom illustrates the antizyme-dependent targeting pathways for ODC.

3.2.1. Ubiquitin-dependent targeting mechanism

Most of the well-characterized proteasomal substrates are tagged by polyubiquitin chains. Ubiquitin is a small protein of 78 amino acid synthesized as different fusion proteins with the 78 amino acids placed N-terminal to small ribosomal peptides. The special "marriage" of ribosomal peptides with ubiquitin has been considered to allow ubiquitin to serve as a molecular chaperon to assist ribosome biogenesis. Ubiquitin is then cleaved from the ribosomal peptide to mark different proteins for different purposes, among which is the targeting of a protein to the 26S proteasome for degradation.

When a protein is damaged, misfolded, or contains distinct "signatures" for rapid turnover, the protein is generally marked by ubiquitin covalently. The formation of the covalent bond between an Ub and a substrate is catalyzed by a set of enzymes in an orderly fashion. The first enzyme, called E1, forms a thioester bond with the carboxyl-group of the carboxyl terminus of the ubiquitin and then transfers the activated Ub onto a second enzyme, E2, which also forms a thioester bond with Ub. The third enzyme E3, which can bind the protein substrate directly or indirectly, mediates the last step of transfer of the Ub from E2 to the epsilon-amino group of an internal lysine residue of the substrate. There is only one E1 in eukaryotic cells, but there are many species of E2s (also called ubiquitin conjugating enzymes) and many more E3s that play the unique role of determining the substrate specificities.

In Hershko and Ciechanover's review article, E3 is defined as "an enzyme that binds, directly or indirectly, specific protein substrates and promotes the transfer of ubiquitin, directly and indirectly, from a thiolester intermediate to amide linkage with proteins or polyubiquitin chains" (43). Four different types of E3 are known: 1) the main N-end rule E3, which is an 200 kDa protein responsible for ubiquitination of N-end rule protein substrates (those containing basic or bulky-hydrophobic N-terminal amino acid residues); 2) the HECT (Homologous to E6-AP C-terminus) family E3, which includes members such as E6AP, Pub1, RSP5 and its mammalian counterpart Nedd4; 3) the multi-subunit complex of cyclosome, also called the APC (Anaphase Promoting Complex) which is known to mediate the ubiquitination of cyclins and several other cell cycle regulated proteins containing a nine-amino-acid motif called "the destruction box"; 4) the SCF (Skp-Cullin-F box) complex ligase, whose substrates are phosphorylated; it is thus also called Phosphoprotein-Ubiquitin Ligase Complexes (PULCs) (details see in reference 43).

The targeting of a protein to the 26S proteasome for degradation usually requires a process called poly-ubiquitination, in which conjugation of one ubiquitin onto another ubiquitin occurs via the formation of the isopeptide bond between K48 of ubiquitin n with the G76 of ubiquitin n+1. Such a polyubiquitination chain has been construed to create a unique structural element that is recognized by the 19S complex (54). However, such a receptor has not yet been identified, although one protein called 5Sa appears to contribute partially to the recognition of the polyubiquitination chains (55). Overall, many questions still remain to be addressed, regarding the mechanisms of the degradation of a polyubiquitinated protein: how is it recognized by the proteasome? How is it unfolded? How are the polyubiquitination chains cleaved? How does the entry of the 20S proteasome open to allow threading in of the substrate?

3.2.2. Antizyme-dependent targeting mechanism

In 1992, Murakami and coworkers reported the ability of a protein called antizyme (AZ) to mark and target the ornithine decarboxylase (ODC) to the proteasome for degradation (40). Although multiple proteins are now known to undergo ubiquitin-independent proteasomal degradation, little is known regarding the underlying mechanisms. So far, the best-studied Ub-independent degradation is still the AZ-dependent ODC degradation.

ODC catalyzes the rate-limiting step in polyamine synthesis. Upon the increase of cellular polyamine level, the translation of AZ is activated via a novel frame-shift mechanism (20;21). AZ then forms a non-covalent complex with ODC, via its C-terminus domain. The N-terminus domain of AZ contains a signal for targeting the complex of AZ/ODC to proteasome for degradation, since deletion of this region completely abolishes the ability of AZ to induce ODC degradation by proteasome (56;57). Like ubiquitin, AZ is recycled after delivery of ODC to proteasome for degradation. Thus, AZ represents a new type of marker for targeting a protein to proteasome. However, prior to the identification of Smad1 as an AZ interactor (see below), ODC remained as an "orphan" for the AZ-dependent proteasomal targeting pathway.

4. PHYSICAL AND FUNCTIONAL INTERACTIONS BETWEEN PROTEASOME SYSTEM AND SMADS

In this section, the interaction between Smads and multiple components along both the ubiquitin-dependent and AZ-dependent proteasomal degradation pathways will be summarized. The first half of the section

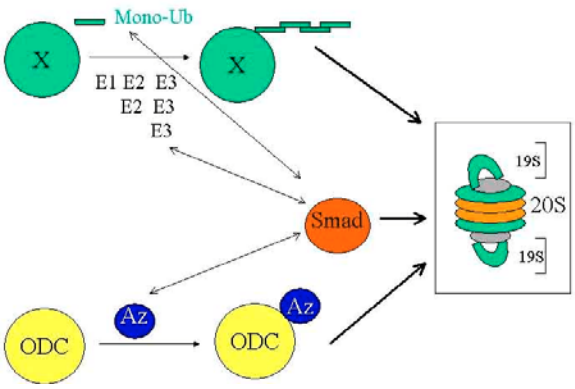

Figure 3. Smad1 interaction with multiple proteins in proteasome-mediated degradation systems. In the ubiquitin-dependent pathway, Smad1 interacts with E3s and ubiquitin; in the antizyme-dependent pathway, Smad1 interacts with antizyme. Smad1 also interacts with HsN3, a beta subunit of the 20S proteasome. Although not depicted in this cartoon, the interaction between Smad1 and HsN3 occurs prior to the assembly of the mature 26S proteasome (10).

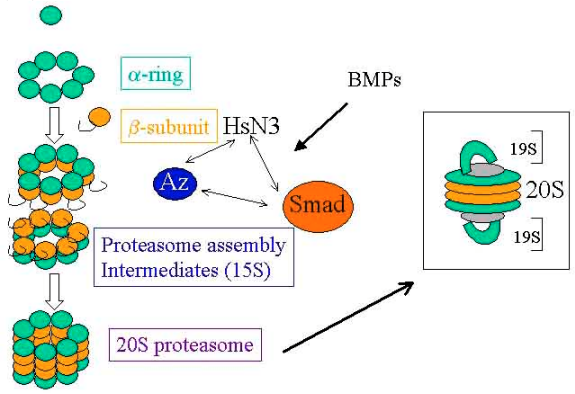

Figure 4. BMP regulates the complex formation of Smad1 antizyme and HsN3 along the proteasome assembly pathway. Details see ref s 10 and 12.

focuses on Smad interaction with substrate markers (Ub, AZ) and the proteasome beta subunit HsN3; the second half focuses upon the various ubiquitin E3 ligases. A summary of the proteasome components that interact with Smad1 is provided in Figure 3.

4.1. Substrate-targeting proteins and the proteasome subunit (UBA52, UBA80, AZ and HsN3)

4.1.1. Physical interaction

In 1996, when the human cDNA of Smad1, then called BSP-1 (TGF-beta Signaling Protein-1), was first cloned, it was noted that BSP-1 could co-precipitate with a large number of endogenous proteins from A549 cells (9). Based upon this data, a collaborative study was carried out to apply the yeast two-hybrid system to identify Smad1 interactors. From screening about 1 million cDNA clones, thirteen different specific interactors for Smad1 were identified (10). Among them, there are four proteins that have clear functional roles in the 26S proteasome-mediated degradation system: two Ub fusion proteins UBA52 and UBA80, the ornithine decarboxylase AZ, and the proteasome beta subunit HsN3.

The interaction between Smad1 and the two UBAs is currently limited to the yeast two-hybrid system. Domain mapping of UBAs showed that the ribosomal peptides on these fusion proteins are neither necessary nor sufficient for the interaction with Smad1 (Lin *et al.* unpublished).

The interactions between Smad1, AZ and HsN3 have been studied in mammalian overexpression systems (10). Smad1 can co-precipitate with AZ when both are co-expressed in 293 cells, albeit weakly. The interaction between Smad1 and HsN3 appears to occur transiently, prior to the complete assembly of HsN3 into the mature 20S proteasome. The latter conclusion is derived from the following observations: 1) Smad1 does not interact with the mature 20S proteasome; 2) Smad1 only co-precipitates with the immature pro-sequence-containing form of HsN3; 3) Smad1 co-precipitates with proteasome assembly intermediates when the assembly of HsN3 is blocked upon artificially removing the prosequence; 4) Smad1 and pro-sequence-containing HsN3 can be co-fractionated along the sucrose gradient (M. Mach *et al.*, unpublished data).

Interestingly, AZ also interacts with HsN3, especially when Smad1 is co-expressed. Domain mapping studies revealed that the N-terminal domain of AZ, which is known to contain the proteasome-targeting signal, binds to HsN3, while the C-terminal domain of AZ interacts with Smad1. In yeast, mutual interactions between Smad1, AZ and HsN3 have been detected. Thus, it is possible that these three proteins can form a ternary complex *in vivo* (10;12).

The interaction between Smad1 and HsN3 is enhanced upon BMP type I receptor activation. The interaction between Smad1 and AZ, however, does not appear to be significantly altered by receptor activation (10).

A cartoon to the left (Figure 4) illustrates the potential ternary complex formation between Smad1, HsN3 and Az along the known proteasome assembly pathways. The ternary complex formation is subjected to the regulation of BMP stimulation (10).

4.1.2. Function

It is currently not clear whether there is a functional role of the observed interaction between Smad1 and the UBAs. Although the ribosomal fragments on these UBAs are dispensable for the interaction with Smads, they might play a regulatory role in the interaction, since different interaction affinities between Smad2 and the two UBAs (UBA52 and UBA80) were detected (10). The interaction between Smad1, AZ and HsN3 has been suggested to mediate the proteasome-assembly-coupled targeting of activated Smad1 to the proteasome (12). In the signaling pathway of BMPs, the activation of Smad1 by the BMP type I receptor triggers the proteasomal degradation

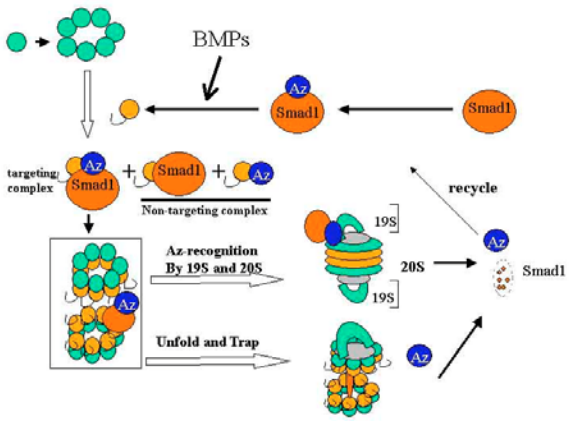

Figure 5. A model for BMP-induced proteasomal targeting of Smad1 to the 26S proteasome for degradation. Smad1 interacts weakly with AZ before BMP stimulation. BMP stimulation triggers the complex formation of Smad1/AZ/HsN3 (HsN3 containing prosequence), which is routed into the proteasome assembly pathway. Smad1 is delivered into the 20S/26S proteasome via two possible ways and then degraded (see text for details). AZ is recycled.

of Smad1. Overexpression of either AZ or HsN3 inhibited the degradation of Smad1 (12), suggesting a role of HsN3 and AZ in regulating BMP-induced Smad1 degradation by the proteasome. Based on biochemical and functional characterizations of the interactions between these three proteins, a model has been proposed (Figure 5), in which interaction between Smad1 and HsN3 prior to HsN3 incorporation into the proteasome allows Smad1 to be delivered to the proteasome. One delivery route could be that described for UMP1, which also binds to the pre-assembled proteasome intermediates and subsequently is possibly unfolded and trapped inside of the proteasome for degradation (The "Unfold and Trap" route) (49). A second route could be via the interaction between Smad1/AZ and a component(s) of the 19S regulator. The interaction between Smad1 and HsN3 could simply serve to bring Smad1 closer to the 19S complex. In either case, excess expression of HsN3 and AZ could lead to the accumulation of complexes that either do not undergo assembly or lack targeting activities (non-targeting complex, as illustrated in Figure 5), thus blocking Smad1 targeting to proteasome. The targeting event is triggered by BMP type I receptor activation, which enhances the interaction between Smad1 and prosequence-containing HsN3.

While it is possible that the complex formation between Smad1, AZ and HsN3 is sufficient to target activated Smad1 to proteasome for degradation, poly-ubiquitination of Smad1 has also been detected in response to BMP type I receptor activation (12). This implies that ubiquitin-dependent targeting of activated Smad1 could also be involved. These two pathways could exist independently or coupled. For example, Smad1 is polyubiquitinated, forms a complex with AZ and HsN3, and then both AZ and the polyubiquitination signals serve to dock Smad1 into the degradation chamber. Such a coupling mechanism could exist for many other Smad-regulated targeting of ubiquitinated substrates. Understanding of the relationship between these two types of targeting mechanisms for Smads is important and awaits future studies. The finding of the functional link between AZ and HsN3 also suggests a role of HsN3 as a proteasomal receptor for AZ during targeting ODC to proteasome. However, the targeting of ODC and Smad1 to proteasome must involve different mechanisms, since Smad1 targeting by AZ is dependent upon the activation of BMP type I receptor, while ODC targeting by AZ appears to be constitutive.

One important extension of the above observations is that the targeting of Smad1 to proteasome is further coupled to the targeting of Smad1 interactors to proteasome for degradation. For example, one nuclear interacting protein of Smad1, called SNIP1 (Smad1 Nuclear Interacting Protein-1), which functions as a constitutive repressor of the master transcription co-activator CBP/p300, is suggested to be co-targeted to proteasome for degradation along with Smad1 (10;58). However, due to the multi-component nature of the complex, many more detailed interaction studies are required to elucidate the biochemical events involved in SNIP1 degradation.

4.2. The E3 ligase family of proteins

4.2.1. The HECT family of E3 ligases

4.2.1.1. Smurf1

4.2.1.1.1. Physical interaction

The *Xenopus* Smad1 was used as bait in a yeast two-hybrid screen, which yielded a new member of the HECT family E3 ligase named Smurf1 (Smad ubiquitination regulatory factor-1) (27), which contains an N-terminal lipid/Ca^{2+} binding domain (C2) followed by two WW domains and a C-terminal HECT domain.

Wild-type Smurf1 was unable to interact with Smad1 even when both are overexpressed. However, upon abolishment of the HECT domain ligase activity by mutating the conserved cysteine residue to alanine (C710A), a stable complex with Smad1 was detected.

The interaction specificity was confirmed by testing Smurf1CA interaction with other Smads. Only Smad1 and Smad5, but not Smad2, Smad3, or Smad4, interact with Smurf1 (27). Recent studies revealed that the two inhibitory Smads (I-Smads), Smad6 and Smad7, are the strongest interactors of Smurf1, among the tested eight Smads (Smads1-8) (30).

Domain mapping studies revealed that the interaction is mediated by the WW domains on Smurf1 and the PPXY motifs within the linker region of the interacting Smads.

4.2.1.1.2. Function

Smurf1 mediates ubiquitination of Smad1, Smad5 and Smad7 upon its co-expression with these Smads in cell lines (27;30). The increased ubiquitination of these Smads are not dependent upon the activation of the R-Smads by BMP receptor and correlates well with reduced

steady state level of these Smads, in mammalian cell lines and in *Xenopus* embryo. Thus, Smurf1 is considered to be an E3 ligase that modulates the constitutive Smad1 and Smad5 protein levels, which may determine the scope of the signaling networks that BMPs trigger in a specific cell. The ability of Smurf1 to interact with Smad6 and Smad7 links Smurf1 to two other important aspects of functions (30). First, Smurf1 regulates the steady state level of Smad7 (30). Smad7 is known to function as a feedback signal to turn off TGF-beta signaling via multiple mechanisms and also to serve as a mediator for INF-gamma down-regulation of TGF-beta signaling (31). Second, smurf1 regulates the steady state levels of TGF-beta type I receptor, after it is recruited to the type I receptor by Smad7 (30). Thus, Smurf1 is important in adjusting the scope of BMP signaling networks by regulating the levels of BMP-responsive R-Smads. It also adjusts the feedback signals from the inhibitory Smads in both the TGF-beta and BMP pathways, possibly to reset the responsiveness of a cell to new ligands.

4.2.1.2. Smurf2

The search for proteins homologous to Smurf1 led to the identification of Smurf2 (27-29;36). Like Smurf1, it has the C2-WW-HECT domain signatures. Different from Smurf1, it has three WW domains. Four different research groups have made separate observations on the biochemical and functional properties of Smurf2.

4.2.1.2.1. Physical interaction

Lin *et al.* demonstrated that Smurf2 interacts with Smad1, Smad2 and Smad3, but not Smad4, when Smurf2 and these Smads were co-expressed in 293 cells. The strongest interaction was detected between Smad2 and Smurf2. In addition, Lin *et al.* reported that the interaction between Smad2 and Smurf2 was enhanced upon TGF-beta stimulation (29). Thus, this study suggests a distinct biochemical property of Smurf2 that is different from Smurf1.

Zhang *et al.* observed slightly different interaction properties of Smurf2 when they did the same type of experiments in COS1 cells. Smurf2 also binds to Smad1, Smad2 and Smad3, but Smad1 was by far the strongest interactor for Smurf2 (28). Thus, under certain *in vitro* conditions, Smurf2 also binds to BMP-Smad.

Kavsak *et al.* reported the interaction studies in 293 T cells. Little interaction was detected between Smurf2 and Smads 1, 2, 4, except Smad7, which binds Smurf2 with high affinity. Such an interaction was also detected in U4A/Jak 1 cells, upon induction of Smad7 expression by IFN-gamma. In this study, Smurf2 was also shown to be recruited to form a ternary complex with the TGF-beta type I receptor (31). As discussed below, such interaction likely mediates the ubiquitination of Smad7 by Smurf2 in the complex with the type I receptor of TGF-beta.

Bonni *et al.* reported the interaction between Smad2 and Smurf2 in 293 T cells and confirmed the enhanced interaction between Smurf2 and Smad2 upon TGF-beta stimulation. The authors extended this observation and showed that Smurf2 was also recruited to form a ternary complex with the Smad2 interactor SnoN, an oncoprotein and transcriptional co-repressor (see below) (36).

The variations of the interaction properties likely derive from the use of over-expression systems. The consensus observation among all groups is that different from Smurf1, Smurf2 interacts strongly with Smad2 upon TGF-beta stimulation. The interaction between Smurf2 with Smad1 and Smad3 are overall weaker and the physiological roles of the interactions need to be confirmed. Like Smurf1, Smurf2 also exhibits the strongest interaction with the I-Smad, Smad7, possibly also Smad6. Such interactions may play roles in mediating ubiquitination of the I-Smads or the type I receptors, when ternary complex of Smurf2, I-Smad and type I receptor is formed.

The interaction domains are mapped within the second and third WW domains of Smurf2 and the PPXY motifs of the Smads.

4.2.1.2.2. Smurf2 functions

Three functional properties of Smurf2 are summarized below:

Regulation of the ubiquitination and degradation of Smad1 and Smad2: Smurf2 acts similarly to Smurf1, in that it adjusts the constitutive levels of Smad1 and Smad2 to regulate the cellular responsiveness to TGF-beta and BMPs (28). The different specificity towards Smad1 or Smad2 observed in separate reports could be cell-specific (28;29).

Regulation of the ubiquitination and degradation of Smad7 and TGF-beta type I receptor: Smurf2, when associated with Smad7, is recruited to and form a complex with the activated TGF-beta type I receptor (31). Within the complex, Smurf2 ubiquitinates Smad7 but not the type I receptor. The ubiquitinated Smad7 is targeted to proteasome for degradation, along with the type I receptor. It was shown that Smurf1 is a nuclear protein and the increase of Smad7 level alone is sufficient to trigger Smurf2 interaction with Smad7. The interaction induces the translocation of Smurf2 from the nucleus to the cytoplasm and the subsequent recruitment to the type I receptor to down regulate the receptor (31). Such a mechanism could be an important cross-talk mechanism by INF-gamma inhibits the TGF-beta pathway (31). Also, the ability of Smad7 to bring Smurf2 to the activated TGF-beta receptor could be an important mechanism by which Smad7 functions as a negative feedback signal.

Regulation of the ubiquitination and degradation of Smad interactors: Smurf2, when associated with Smad2 in response to TGF-beta receptor activation, is further recruited by Smad2 to form a complex with the Smad2 interactor SnoN, an oncoprotein known to function as a nuclear transcriptional repressor (36). In this ternary complex, Smurf2 ubiquitinates SnoN and targets SnoN for proteasomal degradation. Smad2 serves as an adapter for

the E3 ligase Smurf2 to mediate ubiquitination of the substrate SnoN. The ubiquitination and degradation of SnoN directly contribute to the signaling outcome of TGF-beta stimulation. Thus Smurf2 functions, in this case, as an active signaling mediator in TGF-beta signaling pathways.

4.2.1.3. AIL4/hItch

AIP4 (Atrophin-1 interacting Protein 4) is a third C2-WW-Hect family protein, which exhibits specific interactions with Smads (Guedes *et al.*, manuscript in preparation). It was originally cloned as an interactor of Atrophin-1, the protein implicated in the neurodegenerative disease DRPLA (Dentatorubral pallidoluysian atrophy) (23). The mouse counterpart of AIP4 is a protein called Itch, deletion of which leads to the autoimmune phenotype of the *Itch* mice (22). Thus, AIP4 is also named as human Itch (hItch). AIP4/hItch has been shown to be an E3 ligase for JunB (59). The physical link between AIP4/Itch with Smad3 was revealed from a yeast two-hybrid screen using Smad3 as bait (Liu *et al.*, unpublished).

4.2.1.3.1. Physical interaction

The hItch binds to Smad1, Smad2, Smad3, but not Smad4 in the yeast two-hybrid system. Smad3 is the strongest hItch interactor among the three tested R-Smads, but is a weaker interactor when compared with Smad6 and Smad7. The interaction between Smad3 and hItch has been confirmed in mammalian cells. Domain mapping studies revealed that the interaction is dependent upon the second and third WW domains on AIP4/hItch and the PPXY motif within the linker region of Smad3 (S. Guedes *et al.* manuscript in preparation).

4.2.1.3.2. Function

The AIP4/hItch E3 ligase does not ubiquitinate any R-Smads, but can directly ubiquitinate Smad6 and Smad7. However, both I-Smads are not targeted to proteasomal degradation upon their ubiquitination by hItch. AIP4/hItch also ubiquitinates the Smad3 interactor HEF1 upon its co-expression with HEF1 in 293 cells (L. Feng *et al.*, manuscript in preparation). Studies are ongoing to determine whether AIP4/hItch is the functional partner of Smad3 to mediate the ability of Smad3 to regulate the proteasomal degradation of Smad3 interactors such as HEF1 and SnoN.

4.2.2. The Anaphase Promoting Complex

So far two papers have reported the role of APC complex in Smad3-regulated proteasomal degradation of the oncoprotein SnoN (37;38). The APC complex was first identified and purified from the cell-free systems of clam oocyte, based upon its cell-cycle regulated activity of ligating ubiquitin to cyclin B at the end of mitosis (60). Protein purification of the APC complex revealed a large protein complex of approximately 1,500 kDa (61). In mammals, the APC complex consists of 11 core subunits (62). Similar to the SCF complex E3 ligase (see below), the APC complex contains a ring finger protein (APC11), presumably involved in interaction with E2, as well as a protein with Cullin-like domain (APC2). Two WD-40 proteins, CDC20 and CDH1, associate with APC in a cell cycle-regulated fashion to activate APC as a substrate-specific E3 ligase. CDC20 activates APC during mitosis to ubiquitinated proteins containing a nine-residue destruction box (D box: RXXLXXXXN), which is present in proteins such as cyclin B and pds1 (63; 64). CDH1 associates and activates APC in G1 as well as in differentiated cells, thus targeting APC to ubiquitinate both cell-cycle-specific and cell-cycle-unrelated proteins (65). An interaction between Smad3 and the APC complex has been reported and shown to contribute to the ability of Smad3 to regulate proteasomal degradation of SnoN (see below).

4.2.2.1. Physical interaction

Stroschein *et al.* observed that the MH2 domain of Smad3 or Smad2 was sufficient to induce the ubiquitination of SnoN (37). Since these domains cannot interact with Smurf2, the data suggested the existence of another E3 ligase that interacts with the MH2 domains of Smad3 and Smad2. The identification of the essential role of the D box on SnoN for Smad3-regulated SnoN ubiquitination further suggested the involvement of the APC complex. Upon the immunoprecipitation of an overexpressed Smad3 in 293T cells, two important components of APC complex: Cdc27 and Cdc16 were detected, implicating the APC complex (37). Such an interaction was enhanced by the activation of TGF-beta signaling (37). The APC complex interacts with the MH2 domain of Smad3, which also interacts with the N-terminal domain of SnoN (1-97 a.a.). However, the two domains are distinct, since a Smad3 mutant (S3S1S3) with a small region of the MH2 domain (a.a.230-289) replaced by the corresponding region of Smad1 was able to bind to APC but not SnoN, while a Smad1 mutant (S1S3S1) that contains all its original sequence except the exchange of its corresponding domain with that of the MH2 domain of Smad3 (a.a.230-289) was able to bind to SnoN but not to the APC complex (37). The exact Smad3-binding component within the APC complex has yet to be fully characterized. Preliminary data from our lab suggests the direct interaction between Smad3 and the APC10 component (C. Neurry *et al.*, manuscript in preparation). An additional interaction has been detected between CDH1 and the SnoN, but such an interaction appears to require Smad3 and activation of TGF-beta signaling (37).

4.2.2.2. Function

Both *in vivo* and *in vitro* ubiquitination studies revealed that the APC complex ubiquitinates SnoN in the presence of Smad3 (37). Thus, it was suggested that Smad3, via simultaneous interaction with SnoN and APC, serves as an adapter protein to recruit the APC complex to SnoN, allowing the APC ligase to ubiquitinate SnoN (37). SnoN has three regions, mutation of any one of which would block its ubiquitination by APC and its subsequent degradation by proteasome: 1) the N-terminal Smad3 binding site, which has been fine mapped to be within a.a. 89-92; 2) the D-box (a.a. 164-172), which mediates the interaction with CDH1; and 3) the ubiquitination sites (K440, 446, 449).

Since all known APC substrates play important roles in the cell cycle, Wan *et al.* examined SnoN degradation in synchronized Hela S3 cells along the cell

cycle (38). SnoN protein level increases at the onset of G2 and peaks at G2/M, but declines rapidly at the end of M and remains low at G1. Thus, SnoN degradation is subjected to cell cycle regulation. *In vitro* SnoN degradation was reconstituted by using mitotic *Xenopus* egg extracts, which contain the activated APC complex. In this *in vitro* system, Wan *et al.* demonstrated the role of the D-box on SnoN for SnoN degradation, which is shown to be sensitive to proteasome inhibitors. Another *in vitro* system using extracts from TGF-beta treated mink lung epithelial cells was also successfully established to study SnoN ubiquitination and degradation. These *in vitro* systems allowed systematic dissection of different components in the ubiquitination and degradation of SnoN in both cell cycle regulated and TGF-beta induced events. These studies also revealed the essential roles of Smad3, the D-box of SnoN, and the CDH1 protein in mediating SnoN ubiquitination and degradation (38).

4.2.3. The SCF complex

4.2.3.1. Physical interaction

The SCF complex is known to ubiquitinate phosphorylated substrates. Although at the beginning only three subunits were identified for the active ligase (Cullin, Skp, and F-box protein), a fourth subunit called ROC1 (also called Rbx1 or Hrt1) is now known to play an essential role in the E3 ligase activity of SCF complex (66-68). ROC1 is a ring-finger protein Cullin-binding protein which functions as an adapter of the SCF complex to recruit E2-conjugating enzymes. The ROC1-SCF complex (with the F box protein Fbw1a) is responsible for the phosphorylation-dependent ubiquitination of I-kappa Balpha and beta-catenin, thus playing a critical role in regulating these major signal transduction pathways.

Recently, it has been reported the Smad3, via its MH2 domain, interacts with ROC1, in the yeast two-hybrid system (32). The interaction was confirmed by immunoprecipitation in mammalian cells and shown to be induced upon the activation of the TGF-beta type I receptor. Regarding the molecular domains involved in the interactions, the following observations have been made: 1) Smad4 does not interfere with the interaction, suggesting different binding motifs of Smad3 MH2 domain are involved in interaction with Smad4 and ROC1 and 2) on ROC1, mutations within the ring finger domain (C75A/H77A) abolish its interaction with E2, but not the interaction with Smad3 MH2. The interaction is not only regulated by the TGF-beta type I receptor but by the protein levels of other components of the SCF complex. Co-expression of both Cul 1 and Fbw1a greatly enhanced the interaction between Smad3 and ROC1, as well as the ubiquitination of Smad3 (see below). Interestingly, coexpressing p300 also enhances the interaction as well as the ubiquitination. On the contrary, coexpressing the transcriptional co-repressor Ski inhibits the interaction as well as the ubiquitination of Smad3 by the SCF complex. The correlation of the increased Smad3 interaction with ROC1 and the increased Smad3 ubiquitination could suggest a positive role of Smad3 ubiquitination in Smad3 interaction with ROC1.

4.2.3.2. Function

Coexpression of ROC1 with Smad3 did not induce a significant increase of Smad3 ubiquitination; however, a great increase was observed upon coexpression of ROC1, Cul1 and Fbwa1, and TGF-beta type I receptor activation further enhances the ubiquitination. This is consistent with enhanced complex formation by the coexpression of these SCF complex components. The specificity of the effect of the SCF complex on Smad3 ubiquitination was demonstrated by two different approaches: 1) among all tested Smads (Smads1, 2, 3, 4 and 8), only Smad3 and Smad8 were ubiquitinated; 2) of the tested domains of Smad3, only Smad3 MH2, but not Smad3 MH1 and Linker regions, was ubiquitinated; and 3) among three tested F box proteins, only Fbwa1 enhanced Smad3 ubiquitination. Consistent with the observed opposite effects of p300 and c-Ski on the complex formation between Smad3 and ROC1-SCF complex, these two proteins also exhibit opposite effects on Smad3 ubiquitination. The ubiquitinated Smad3 is degraded by proteasome, since proteasome inhibitors such as MG132 and lactacystin can stabilize the Smad3 complex as well as the level of ubiquitinated Smad3. Interestingly, the localization studies revealed that the co-expression of the ROC1-SCF complex with Smad3 MH2 induces the nucleus-to-cytoplasm translocation of Smad3 MH2. The translocation of Smad3 MH2 is dependent upon the integrity of the ROC1-SCF complex, since either lack of ROC1, Cul1, or Fbwa1, or mutations of Cul1 or Fbwa1, can abolish the translocation. It is not clear whether ubiquitination of Smad3 occurs before or after the nucleus-to-cytoplasm translocation.

4.3. The COP9 signalosome

Genetic studies of the light-mediated signaling pathways in plant *A. thaliana* led to the identification of the protein complex COP9, which contains eight subunits, all of which possess homologies with the eight non-ATPase subunits of the lid complex of the 19S regulator within the 26S proteasome. The mammalian counterpart of the COP9 complex was also purified and characterized. Due to its ability to regulate the phosphorylation of several key signal transducers, such as I-kappa B alpha, c-Jun, and p105, the COP9 complex has been renamed the COP9 signalosome (69;70).

4.3.1. Physical Interaction

One of the COP9 signalosomes, CSN5, also named Jab1, was isolated in a yeast two-hybrid screen as a specific interactor for Smad4. Jab1 was initially identified as the Jun activating domain binding protein and also has been shown to induce proteasomal degradation of p27 and p53 (71-73). The interaction between Smad4 and Jab1 was mediated by the MH2 and linker region of Smad4. A Jab-binding consensus domain found in p27, p53, and c-Jun was also found within the Smad4 MH2 domain. It was suggested that the linker regions of Smad4 contains a putative phosphorylation site for the COP9 signalosome

(74). Whether the entire COP9 signalosome interacts with Smad4 remains to be elucidated.

4.3.2. Function

Smad4 was shown to be ubiquitinated and degraded by the 26S proteasome upon the co-expression with Jab1. Consistent with this observation, overexpression of Jab1 efficiently inhibited the gene activation responses of TGF-beta in Mv1Lu cells (74).

5. A SUMMARY OF PROTEASOME-MEDIATED DEGRADATION IN TGF-BETA FAMILY-MEDIATED SIGNALING EVENTS

5.1. Proteasome-mediated degradation in the down-regulation of Smads and the activated type I receptor

5.1.1. Ligand-independent regulation of the protein levels of Smads

The protein levels of R-Smads are subjected to the ligand-independent regulation by 26S proteasome-mediated degradation. Two HECT family E3 ligases have specific activities towards specific Smads. Smurf1 appears to target primarily Smad1 and Smad5, while Smurf2 specifically targets Smad2 (27-29).

The protein levels of the co-Smad, Smad4, are regulated by Jab1-induced ubiquitination and subsequent degradation by proteasome (74), While the protein levels of anti-Smads, Smad6 and Smad7, are regulated by Smurf1-induced ubiquitination and subsequent proteasomal degradation (30).

No E3 ligase has yet been identified to regulate the constitutive level of Smad3 or Smad8.

While the above studies concern the regulation of the constitutive levels of wild type Smads, proteasome-mediated degradation has also been reported to contribute to the inactivation of mutant Smads associated with cancers (75;76).

5.1.2. Ligand-dependent regulation of the protein levels of Smads

In TGF-beta induced signaling pathways, Smad2 and Smad3, in their activated forms, are both subjected to proteasome-mediated degradation, but perhaps via different mechanisms. The TGF-beta type I receptor-activated Smad3 is ubiquitinated upon forming a complex with ROC1-SCF^{Fbwa1} complex and is then targeted to proteasome for degradation (32). The degradation has been suggested to occur upon nucleus-to-cytoplasm translocation. The TGF-beta type I receptor-activated Smad2 is also ubiquitinated, but the E3 ligase for the activated Smad2 has not yet been determined (33). The ubiquitination and degradation of activated Smad2 is not sensitive to an inhibitor leptomycin B, which blocks the nucleus-to-cytoplasm translocation, thus the degradation of Smad2 is considered to occur inside of the nucleus (33;77).

In BMP-induced signaling pathways, activated Smad1 is also ubiquitinated and targeted to the proteasome for degradation. The E3 ligase for activated Smad1 is not shown. It is also possible that the targeting of activated Smad1 to the proteasome involves both ubiquitin-dependent and AZ-dependent mechanisms. It is possible that the ubiquitin-dependent and AZ-dependent pathways are coupled to mediate the targeting of activated Smad1, as proposed by Gruendler *et al.* (12). The targeting of activated Smad1 to the proteasome involves a complex formation between Smad1, AZ and the proteasome beta subunit HsN3. All three proteins, along with Smad4, are translocated into the nucleus upon the activation of BMP signaling pathways. In the nucleus, the complex of Smad1, Smad4, HsN3 and AZ further associates with CBP/p300 and the CBP/p300 repressor SNIP1. SNIP1 is then targeted for proteasomal degradation together with Smad1 (10).

5.1.3. TGF-beta-induced proteasomal degradation of the TGF-beta type I receptor

Two groups have reported the role of the anti-Smad, Smad7, in recruiting the HECT family E3 ligases Smurf1 or Smurf2, to the TGF-beta type I receptor, triggering ubiquitination of Smad7 (31) and TGF-beta type I receptor (30), and their subsequent degradation by the proteasome as well as the lysosome. Smad7 is therefore functioning as an adapter for the Smurfs to approach the substrate, the type I receptor. It was noted that Smurf2 was unable to ubiquitinate the type I receptor, but can ubiquitinate Smad7 in the presence of the type I receptor (31); in contrast, Smurf1 was able to ubiquitinate Smad7 on its own, and can ubiquitinate the type I receptor in the presence of Smad7 (30). Whether the reported differences from two groups reflect a true different property of these two Smurfs needs further evidence. It was suggested that Smad6 also works with Smurf2 to down-regulate the activated BMP type I receptor (31). The ability of Smad7 to regulate receptor ubiquitination and degradation may contribute to Smad7-mediated down-regulation of TGF-beta signaling as well as the cross-inhibition of TGF-beta signaling via IFN-gamma, which up-regulates Smad7 levels (77).

5.2. Proteasome-mediated degradation in Smad-mediated cytoplasmic and nuclear signaling

5.2.1. Proteasomal degradation of SnoN and Ski

SnoN and Ski are related oncoproteins and can function as transcriptional co-repressors by recruiting the N-CoR and histone deacetylase (HDAC) transcriptional co-repressor complex to specific gene promoter, such as those of the TGF-beta responsive genes (34; 78-80). Both proteins interact with Smad2, Smad3 and Smad4 in a TGF-beta-dependent fashion and can inhibit TGF-beta responsive promoters such as c-Myc (79). It was initially thought that the interaction between Smad3 and these proteins was to mediate transcriptional repression of Smad3-responsive genes, but the story took an interesting turn when it was found that the interaction actually induces rapid proteasomal degradation of Ski and SnoN (34;35). Thus, upon TGF-beta stimulation, the Smad3/Smad4 complex enters the nucleus, where Smad3 interacts with the nuclear Ski or SnoN to regulate their ubiquitination and degradation by proteasome, thereby de-repressing the transcriptional activities of these oncoproteins on specific gene promoters. The degradation of SnoN is also followed

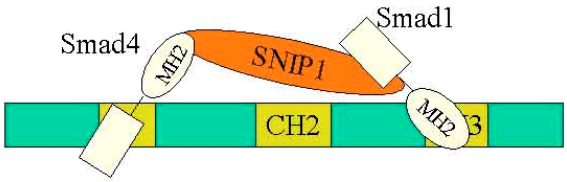

Figure 6. A potential complex between Smad1, Smad4, SNIP1 and CBP/p300. See text for details.

by a rapid transcriptional activation of SnoN gene itself, leading to a rapid recovery of SnoN protein level, as a negative feedback mechanism to control Smad3-mediated signaling (34). From this perspective, Smad3-regulated proteasomal degradation of Ski and SnoN is a downstream signaling event of Smad3 and serves as a mechanism for Smad3 to cross talk to other signaling pathways that are Ski and SnoN-dependent.

5.2.2. Proteasomal degradation of the CBP/p300 repressor SNIP1

SNIP1 was identified as an interactor of Smad1 and Smad2 from the yeast two-hybrid system. SNIP1 forms a stable complex with the transcriptional co-activator CBP/p300 and inhibits its transcriptional co-activator function (58). Such an inhibition could be mediated by the ability of SNIP1 to bind to the C/H1 domain of CBP/p300, thereby blocking the recruitment of CBP/p300 by other transcription factors via the C/H1 domain (58;81; Fluri D., unpublished).

Since SNIP1 interacts with CBP/p300 constitutively, it is likely that a transcription factor must overcome the inhibitory interaction between SNIP1 and CBP/p300. In BMP signaling pathways, activated Smad4 and Smad1 enter the nucleus to activate gene expression, but how Smad1 and Smad4 recruit SNIP1-bound CBP/p300 is not known. Current data suggests the model in Figure 6. The activated Smad4 approaches and interacts with the C/H1 domain via its SAD domain, while its MH2 domain binds the N-terminal domain of SNIP1 (1-141), which binds to the C/H1 domain of CBP/p300 before Smad4 enters the nucleus (58). Thus, Smad4 lifts SNIP1 off from CBP/p300, but it becomes associated with SNIP1. The activated Smad1 interacts with the C/H3 domain of CBP/p300 via its MH2 domain, but interacts with SNIP1 via its linker region (10). Thus, Smad1 and Smad4 could recruit both CBP/p300 and SNIP1 into their DNA-binding complex and then mediate transcriptional activation.

However, the above-mentioned complex of Smad1, Smad4, SNIP1 and CBP/p300 is likely not stable (10). Upon BMP stimulation, Smad1 binds to AZ and HsN3 and brings both of these proteins into the nucleus. SNIP1 also interacts with AZ and HsN3 in yeast two-hybrid systems. Furthermore, the degradation of SNIP1 was observed in response to the activation of BMP type I receptor. Interestingly, SNIP1 degradation is regulated by the protein levels of the activated BMP type I receptor, or Smad1, or Smad4, or AZ. A mutant inactive Smad1 blocked SNIP1 degradation, suggesting that Smad1 activation is necessary for regulating SNIP1 degradation.

It is currently not clear whether ubiquitination of SNIP1 occurs transiently prior to SNIP1 degradation. Although the degradation of SNIP1 is sensitive to proteasomal inhibitors, the inhibition was not complete, suggesting the involvement of additional proteases or novel proteasomal activities (10).

Since SNIP1 is a constitutive inhibitor of CBP/p300, the Smad1- and Smad4-regulated degradation of SNIP1 could assist the recruitment of CBP/p300 by other transcription factors, thus providing a molecular mechanism for Smad1 and Smad4 to work as transcriptional modulators and for BMP signaling pathways to cross talk with other CBP/p300-dependent pathways. Smad4 is well known for its role as a co-Smad for R-Smads in transcriptional regulation, but the molecular mechanisms underlying this activity is not understood. The requirement of Smad4 in Smad-regulated SNIP1 degradation provides an explanation for the functional partnership of Smad1 and Smad4 in mediating CBP/p300-dependent transcriptional activation. This also implies that the ability of Smad4 to cooperate with other R-Smads to regulate the proteasomal degradation of R-Smad binding transcriptional factors could be a common mechanism by which Smads function as transcriptional modulators.

5.2.3. Proteasome-mediated degradation of HEF1 (Human Enhancer of Filamentation)

Cellular attachments to the extracellular matrix component (EMC) send important signals inside of the cell to influence intracellular signaling events that regulate proliferation, differentiation and apoptosis. One of the best-known sensors of environmental cues for attachment and migration is the integrin receptor, clustering of which upon cell adhesion to EMC initiates the formation of focal adhesion complexes. Important cell activities such as cell migration, mitosis, transformation and apoptosis are all dependent upon the dynamic modulation of the focal adhesion components (see review) (82).

In a search for novel regulators of cytoskeletal/oncogenic signaling in mammals, Golemis and her colleagues screened for mammalian proteins that regulate pseudohyphal growth of diploid yeast. This discovery-driven approach led to the identification of the Human Enhancer of Filamentation 1 (HEF1) (83). Morimoto and his colleagues also cloned HEF1, which they called Cas-L, via a biochemical approach, based upon its sequence homology with p130Cas (84). Both p130Cas and HEF1 are key components of focal adhesion complexes. HEF1 is predominantly expressed in epithelial cells and lymphocytes, while p130Cas is expressed ubiquitously, but most predominant in fibroblast cells.

Besides their role in focal adhesions, current evidence suggests that both HEF1 and p130Cas function as multi-domain docking proteins for a large variety of signaling pathways, such as those of TCR-, BCR- and G-protein coupled receptor (GPCR)-mediated signaling. The signaling functions of HEF1 are mediated by its various subdomains. Like P130Cas, HEF1 has an N-terminal SH3 binding domain, followed by multiple Crk-SH2 binding

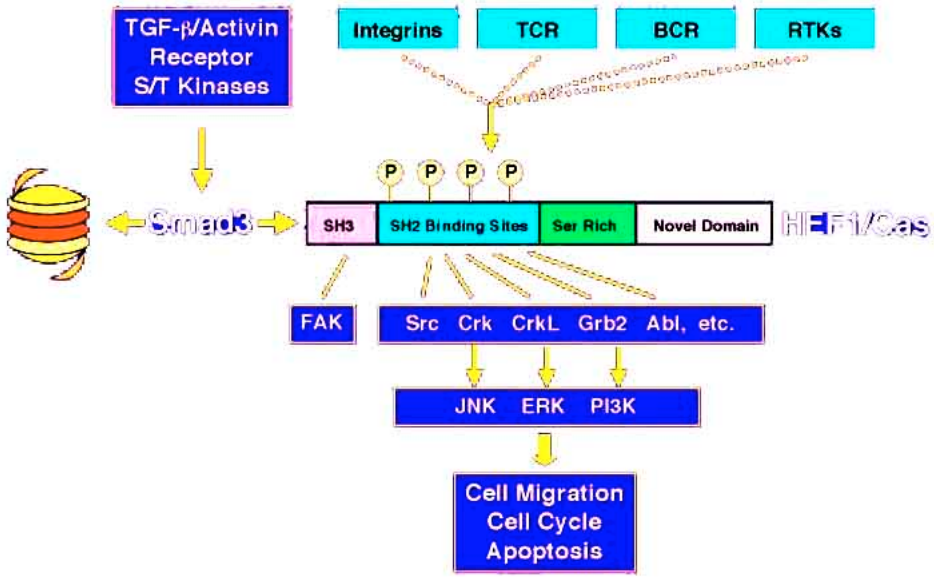

Figure 7. The multi-domain docking protein HEF1 serves as the physical link between multiple signaling events from membrane receptors to multiple signaling events in the cytoplasm and in the nucleus, coordinating cell shape, migration, attachment with cell cycle, differentiation and apoptosis. See Text for details.

sites, a serine rich domain and a C-terminal novel domain containing an HLH motif. The N-terminal SH3 domain of HEF1 partially mediates interaction with the C-terminal poly-proline domain of FAK. HEF1 has 13 repeated YXXP motifs (7YDXP), phosphorylation of which induces the recruitment of various SH2-domain proteins such as Abl, Crk, Nck, SHPTP2, Lck and Csk (85). These proteins further recruit kinases, which induce the activation of various kinase-signaling cascades, such as those of MAPK, JNK and PI3K, leading to the regulation of many different cellular functions (Figure 7).

Because of the dual roles of HEF1 in focal adhesion and signaling, HEF1 likely plays a key role in coordinating cell shape, adhesion and migration with those of cellular responses to various extracellular non-ECM stimuli. To meet such a dynamic role, it is not surprising that HEF1 protein is subjected to all different types of post-translational modifications, which include: phosphorylation, ubiquitination, proteasome-mediated degradation and caspase-mediated cleavage (82; 86-88). The full length HEF1 has two different forms: p115HEF1 and p105HEF1, each containing different phosphoserine and phosphothreonine sites (11). The protein levels of both forms are regulated in response to serum starvation as well as along the cell cycle (86). Interestingly, HEF1 is cleaved by caspase at three caspase sites and produce three different forms, two of which have been well characterized to play distinct functions in regulating cell rounding during mitosis and apoptosis (82; 86-88).

The clue for a functional link between HEF1 with TGF-beta signaling pathways came from a yeast two-hybrid screen using Smad3 as bait. HEF1 was isolated as a specific interactor for Smad3 (11). In 293 cells, transfected HEF1 was expressed as both p115HEF1 and p105HEF1 forms, but Smad3 interacts predominantly with p115HEF1. The interaction was only clearly detectable upon the stabilization of HEF1 by proteasome inhibitors such as lactacystin, since p115HEF1 was rapidly degraded by proteasome. Proteasomal degradation of p115HEF1 was enhanced upon the activation of TGF-beta or activin type I receptors. Domain mapping studies revealed that Smad3 interlocks with HEF1 via their N-terminal and C-terminal domains. However, intramolecular interactions of both proteins may regulate the intermolecular interaction under physiological conditions. This was suggested by the observation that the N-terminal domain of HEF1 (1-505) binds to full length Smad3 while the C-terminal domain of HEF1 can only bind to the isolated MH2 domain but not full length Smad3. Since MH1 and MH2 domains of un-phosphorylated Smad3 interact with each other, it is possible that the interaction between un-phosphorylated Smad3 and HEF1 occurs as a sequential event, in which HEF1 N-terminal domain first recognizes the MH1 domain of Smad3, opening the interaction site on MH2 to be further recognized by the C-terminal domain of HEF1. The interaction between the N-terminal domain of HEF1 (1-505) and the MH1 domain of Smad3 is essential for Smad3 to induce HEF1 degradation, since the degradation can be blocked by the overexpression of N-terminal 505 amino acids of HEF1. The molecular mechanism, by which Smad3 induces HEF1 degradation, is not fully understood. HEF1 is constitutively ubiquitinated. Future studies will determine whether Smad3 interaction with E3 ligases such as Smurf2, AIP4/hItch, SCF, or the APC complex contributes to its ability to induce HEF1 degradation.

The proteasome-mediated degradation of HEF1 in response to TGF-beta was examined and detected in epithelial cell lines such as A549 and HaCaT cells and in T lymphoid cell line T9, with much rapid reduction of HEF1 observed in the epithelial cell lines. Both p115HEF1 and p105HEF1 levels were reduced. Since p105HEF1 can be

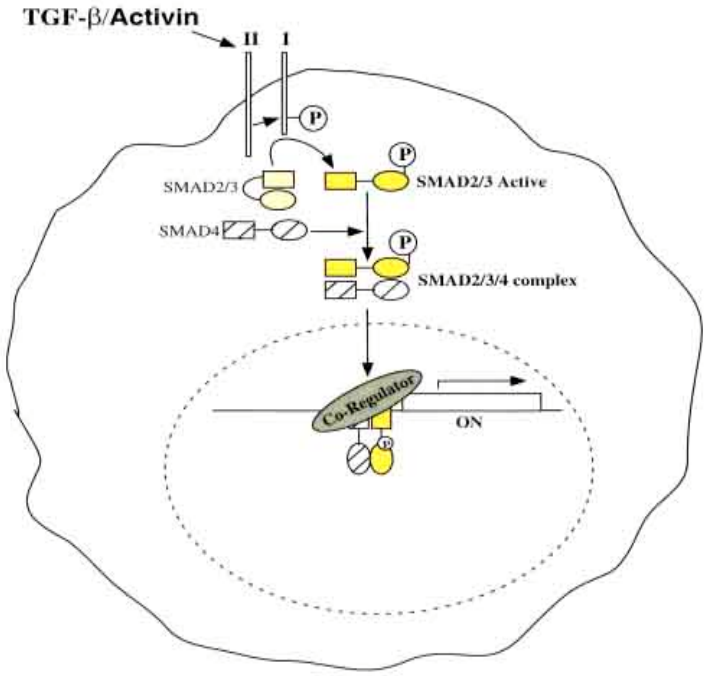

Figure 8. A linear signaling pathway depicting the core signaling events of TGF-beta and activin from cell membrane into the nucleus, via Smad family signal transducers.

converted to p115HEF1, it is not clear whether p105HEF1 was first converted to p115HEF1 before it was degraded. In A549 cells, the reduction of HEF1 level was immediately followed by a rapid actinomycin-sensitive increase of HEF1 level, which was confirmed by Northern blot to be associated with increased mRNA level of HEF1. Thus, TGF-beta induces rapid proteasomal degradation of HEF1, whose level is subsequently restored and even increased to a level surpassing the original level, via the transcriptional activation of HEF1 gene. One of the consequences of such an increase in HEF1 protein level is suggested by the ability of increased HEF1 expression to inhibit TGF-beta induced, Smad3-mediated transcriptional activation of the CAGA-luc reporter gene (11). This implies that the post-proteolytic increase of HEF1 protein level could serve as an efficient feedback mechanism to turn off the transcriptional activity of Smad3 in the nucleus. As mentioned above, a similar phenomenon was observed for Smad3-induced SnoN degradation (34). The increased level of SnoN could directly function in the nucleus to inhibit Smad3-mediated transcription. The molecular mechanism for HEF1-mediated inhibition of Smad3 is not known. Another consequence of increased HEF1 level is to induce apoptosis (87). It is known that TGF-beta can induce apoptosis in some epithelial cell lines, but not in fibroblast cell lines. Considering the epithelial cell-specific expression of HEF1, the increased HEF1 protein levels after prolonged TGF-beta treatment could contribute to the specific pro-apoptotic activity of TGF-beta in epithelial cells. A third consequence is the apparent impact of changing HEF1 protein level on the many signaling pathways involving HEF1, such as integrin, TCR, BCR and calcitonine. This will be further discussed below.

6. PERSPECTIVES: NEW CONCEPTS IN UNDERSTANDING THE MYRIAD BIOLOGICAL ACTIVITIES OF THE TGF-beta SUPERFAMILY

Smads, as key signal transducers of the large TGF-beta superfamily of growth inhibitors, differentiation factors and morphogens, have been primarily recognized as receptor-activated, DNA-binding transcriptional regulators. The core events for Smad-mediated transcriptional regulation are illustrated in Figure 8. In the past several years, proteasomal degradation of Smads has been considered as ways to turn off the signaling pathways (such as the proteasomal degradation of activated Smad2) or ways to adjust the cellular signaling competence (such as Smurf1-regulated proteasomal degradation of Smad1 and Smad5). However, the above outlined physical and functional interactions between Smads and the proteasome system and the ability of Smads to regulate the proteasomal degradation of multiple cytoplasmic (such as HEF1) and nuclear key regulators (such as SnoN, Ski and SNIP1) greatly challenges the paradigm of nucleus-to-cytoplasm linearity of Smad signaling. Below, I summarize several newer concepts regarding Smad-mediated signaling mechanisms.

6.1. Smads are novel regulators of substrate targeting for proteasome-dependent degradation pathways.

There are several mechanisms used by Smads to regulate substrate targeting to the proteasome:

6.1.1. Smad1/HsN3/AZ complex serves as a targeting complex to bring Smad1 interactors to proteasome

As illustrated in Figure 9, in the signaling pathways of BMPs, the BMP-induced complex formation

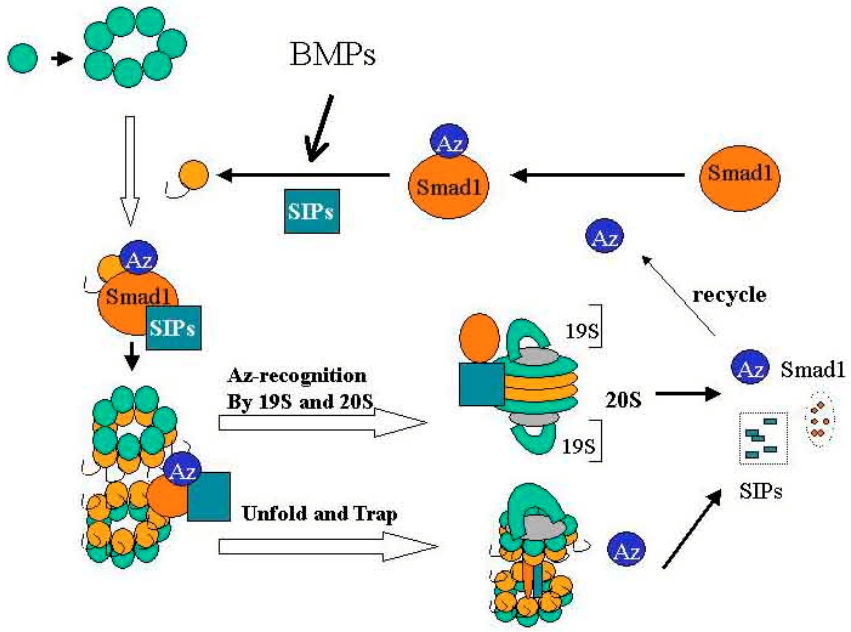

Figure 9. A model for the BMP-induced targeting of Smad1 interactors to proteasome for degradation via the complex formation of Smad1, antizyme (AZ) and prosequence-containing HsN3 along the assembly pathway of 20S proteasome. SIPs: Smad1 Interacting Proteins. The association of Smad1 with HsN3 and AZ allows SIPs to be delivered inside of the 20S/26S proteasome via two possible means, resulting in the degradation of SIPs.

between Smad1, AZ and the proseqeunce-containing HsN3, as explained earlier in Figure 5, could serve as a proteasome-targeting complex which brings Smad1 interacting proteins to the proteasome for degradation via two different mechanisms (10, 12). So far, two interactors originally isolated from the yeast two-hybrid screen appeared to be targeted to proteasome for degradation (Wang T., unpublished data). Such strategy could apply to Smads in TGF-beta pathways, as illustrated in Figure 10.

6.1.2. Smads function as ancillary proteins for various E3 ligases to regulate the ubiquitination of Smad interaction proteins

As summarized in Figure 11, Smads interact with three types of Ub E3 ligases to function as ancillary proteins for these E3 ligases to ubiquitinate different Smad interacting proteins. There are large numbers of Smad1 and Smad3 interactors, which are potential substrates of Ub E3s. The TGF-beta family ligand-induced, Smad-regulated degradation of these Smad3 interactors allows TGF-beta family ligands to cross talk broadly with many different signaling pathways.

6.2. Smads can mediate rapid cytoplasmic signaling without entering into the nucleus

Smad3 regulates the protein level of HEF1, thereby altering HEF1-involved multiple cytoplasmic signaling events and cell morphology (Figure 12).

6.3. Smad-regulated proteasomal degradation of Smad nuclear interactors allow Smads to function as master transcriptional modulators

The ability of Smad1 and Smad4 to regulate co-operatively the degradation of SNIP1, which is a constitutive repressor of the master transcriptional co-activator CBP/p300, points out the ability of these Smads to function as master transcriptional modulator for many transcription factors that recruit CBP/p300 via C/H1 domain. By freeing CBP/p300 from the inhibitory binding of SNIP1, Smad1 and Smad4 could enhance the transcriptional activities of other transcription factors. Since Smad2 also binds to SNIP1, a similar mechanism could also be used by Smad2, Smad3 and Smad4 in TGF-beta and activin pathways to mediate transcriptional regulation (Figure 13).

6.4. TGF-β superfamily signaling: a large signaling network instead of a linear signaling pathway

Recent studies from many research groups have demonstrated cross talk between TGF-beta family signaling pathways and those of many growth factors and cytokines, such as IL2, IL4, IL12, IL6, TNF-alpha, IL1 and IFN-gamma (77;89-94). The functional links between Smads and multi-functional cytoplasmic regulators such as HEF1 and with nuclear regulators such as SNIP1 further point out the complex network of different signaling pathways connected with Smads, as illustrated in Figure 14. The intimate physical and functional connections between the Smad family signal transducers and the substrate-targeting systems of the 26S proteasome (shown in Figures 9-11) suggest that the cross talk could be mediated via the ability of Smads to bring components of each of these pathways to proteasome for degradation. From this perspective, TGF-beta family ligands, which are key regulators of early embryogenesis, function as system managers to coordinate signaling networks of the whole cell by connecting to the proteasome system (Figure 14).

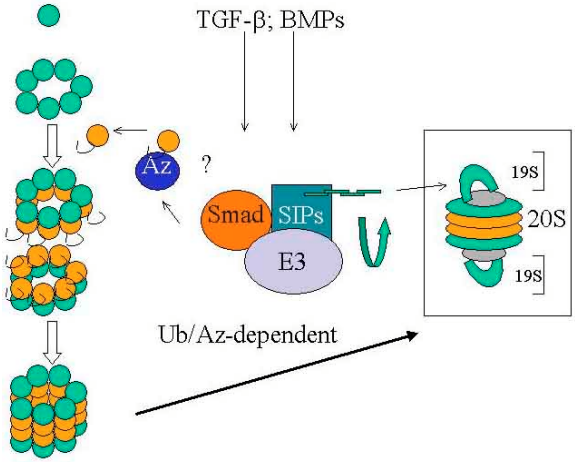

Figure 10. A model for the coupling of the Smad-regulated ubiquitination of Smad interactors with the Smad/AZ/HsN3-mediated proteasomal targeting events. Upon ubiquitination of the SIPs by Smad-recruited E3s, Smad could bring the ubiquitinated SIPs to form the complex of Smad/SIPs/AZ/HsN3 and target SIPs to proteasome along the assembly pathway of the 20S proteasome. Both Antizyme and the polyubiquitination signals could mediate the final docking of SIPs into the degradation machinery.

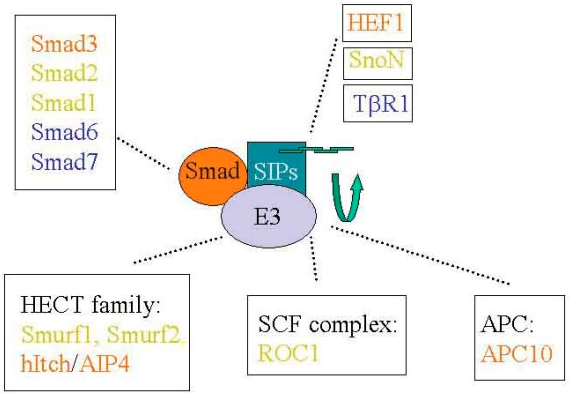

Figure 11. The novel functions of Smads in assisting different Ub E3 ligases to ubiquitinate proteins with which Smads directly interact. The cartoon summarizes the known R-Smads and anti-Smads that function as ancillary proteins for three different types of Ub E3 ligases to mediate ubiquitination of three Smad interacting proteins.

6.5. The functional link between Smad family tumor suppressors and the 26S proteasome suggests an intimate link between proteasome functions and carcinogenesis

Twenty years ago, the 26S proteasome system was regarded as a "garbage dump". Now proteasome is linked with the regulation of almost every aspect of cellular function, from cell cycle control, transcription regulation and various steps of signal transduction to antigen presentation in the immune system and life/death decisions of neurons. While previously the 26S proteasome system was known to be "called in" for some specific work in different signaling pathways, the connection between Smads and the proteasome system summarized in this review further elucidates novel modes of operation of this multi-functional system. Smads, on one hand, are key signal transducers of the TGF-beta family ligands and are directly modified by the receptor kinases. On the other hand, Smads are integral components in targeting proteasomal substrates. The targeting properties of Smads are based upon three unique biochemical properties of Smads: 1) the ability to interact specifically with various E3 Ub ligases and with two types of substrate-marker proteins Ub and AZ; 2) the ability to interact directly with components of the degradation machinery, such as HsN3; 3) the ability to interact with a large number of proteins, mostly via the MH2 domains. Why Smads have so many interactors is not apparent. One possible explanation is the structural similarity between the MH2 domain and the FHA (Forkhead Associated domain), which recognizes phospho-threonine residues (95). Thus, Smad family proteins could be a family of phosphothreonine sensors that link phosphorylation to protein degradation by proteasome. For the TGF-β superfamily, the 26S proteasome is not "called in", but is a "Master Signaling Engine" with which this family of ligands execute their functions as "System Managers" to adjust the signaling levels of a myriad of cellular pathways.

Since multiple Smad family proteins are tumor suppressors, the proteasome link of Smads suggests a critical link between regulated proteasomal degradation and carcinogenesis. At the molecular level, the 26S proteasome has a very unique position in the micro-cosmo of a cell. First, it is responsible for safeguarding normal protein functions by disposing misfolded or damaged proteins. Second, it serves a critical role in adjusting the protein levels of various key regulators thereby altering the activity levels in different systems. That is to say, proteasome links protein metabolism with all aspects of cellular functions. A hyper-metabolic state is commonly observed in cancerous cells, although a functional link of this state to carinogenesis has not been addressed.

In the past twenty years, extensive studies of the cellular and molecular mechanisms of carcinogenesis have led to the understanding that a number of sequential steps leading to accumulated protein mishaps, eventually evading major safeguard mechanisms including the immune system, leading to uncontrolled cell division and metastasis.

The great mystery is: how does a cell manage to accumulate so many mistakes without being eliminated? Genetic instability has been proposed to cause large-scale mistakes at the gene level of a cancerous cells (96). However, genetic instability must also start from a single or pair of genetic mistakes. We now know that a normal cell has sophisticated safeguard mechanisms to detect and repair genetic mistakes and also can activate death programs when it fails to fix the problem. Even if a cell could not fix its own problems, the body has many different ways to safeguard itself and eliminate bad cells. In fact, a human body is immersed in an environment that can constantly introduce mutations within the genetic material,

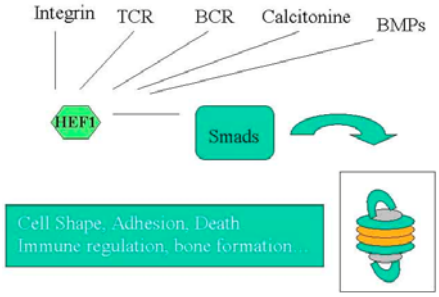

Figure 12. A model by which Smads mediate cytoplasmic signaling events via targeting the cytoplasmic multi-domain docking protein HEF1 to proteasome for degradation. HEF1 is known to signal downstream of many membrane receptors, as illustrated. The rapid degradation of HEF1 upon Smad activation allows rapid adjustment of cellular responsiveness to many other HEF1-involved signaling pathways, thereby broadly regulating cellular functions in a coordinated manner.

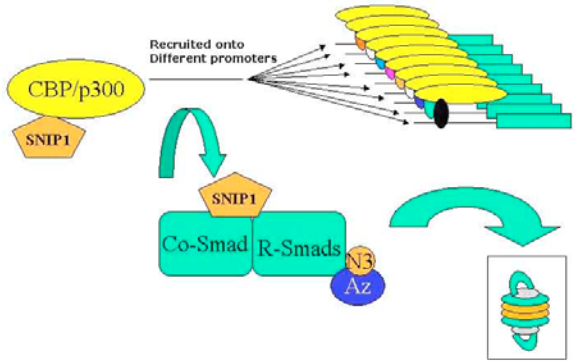

Figure 13. A model for Smads to modulate broadly CBP/p300-dependent transcriptional activation of many transcription factors. See text for details.

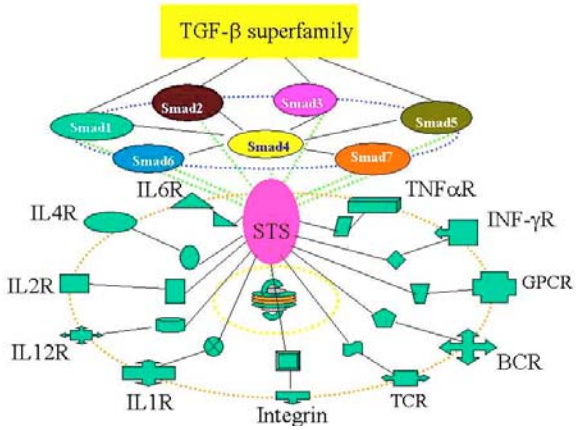

Figure 14. A "system view" of the role of the TGF-beta superfamily ligands in regulating intracellular protein activities. TGF-beta superfamily ligands are system managers to coordinate signaling networks in the whole cell. The listed various pathways likely have multiple connections with the TGF-beta signaling pathways. Many of these pathways involve CBP/p300-mediated transcriptional activation; therefore they are candidate target pathways of Smads via the SNIP1 link. STS: Substrate Targeting System. Here STS refers to the two substrate targeting pathways connected with Smads: the ubiquitin-dependent pathway involving E3 ligases and the antizyme-dependent pathway involving Smad/AZ/HsN3 complex.

but most people do not have cancer. This suggests that the human body normally has the potential to combat the mutations. Therefore, the phenomenon of carcinogenesis is a sign of a system failure, instead of a simple genetic problem.

Then what causes the system failure? Based upon the current knowledge of the unique role of the 26S proteasome in protein metabolism and in regulating and participating in the signaling networks of the large TGF-beta superfamily of tumor suppressors, a model could be proposed to account for what underlies the system failure during carcinogenesis. In this model, excessive hyper cellular metabolism would lead to the over-loading in the capacity of the 26S proteasome, which then fails to function in regulating and participating signaling networks of the tumor suppressors. The combined effects of the accumulation of dysfunctional proteins and the failure of the functions of the Smad family of tumor suppressors can lead to rapid malfunctions of many systems in a cell, including the multiple safeguard mechanisms. If only a few cells have excessive hyper metabolism, then system level safeguard mechanisms should still be able to detect malfunction and eliminate the cells before they form a large mass, or limit its growth and metastasis. However, if the excessive hyper-metabolism occurs in a system fashion, then the cells in the entire system would be under "crisis" and fail to provide safeguard functions. Under such conditions, carcinogenesis would occur at a system level, leading to the failure of the entire body. The excessive hypermetabolic state of large numbers of cells in multiple systems can be induced by emotional and physical stress, which is sensed via the neuro-endocrine systems and manifests the stress as alterations of cytokine levels (97). Here potentially is an important link between carcinogenesis and stress, a mind-body concept known to ancient Chinese and now is beginning to be recognized again by modern people. The complexity of the functional mechanisms of the Smad family tumor suppressors and their intimate functional link with the proteasome point out a new but challenging paradigm for understanding complex system diseases such as cancer.

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