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Smad proteins mediate transforming growth factor-ß (TGFß) signaling to regulate cell growth and differentiation. SnoN is a negative regulator of TGFß signaling that maintains the repressed state of TGFß target genes in the absence of ligand. Upon TGFß stimulation, Smad3 and Smad2 translocate into the nucleus and induce a rapid degradation of SnoN, allowing activation of TGFß target genes. We have shown that Smad2- or Smad3-induced degradation of SnoN requires the ubiquitin-dependent proteasome and can be mediated by the anaphase promoting complex (APC) and the UbcH5 family of ubiquitin conjugating enzymes. Smad3 and to a lesser extent, Smad2, interact with both the APC and SnoN, resulting in the recruitment of the APC to SnoN and subsequent ubiquitination of SnoN in a destruction box-dependent manner. In addition to the destruction box, efficient ubiquitination and degradation of SnoN requires the Smad3 binding site in SnoN as well as key lysine residues necessary for ubiquitin attachment. Mutation of the lysine residues results in stabilization of SnoN and in enhanced antagonism of TGFß signaling. Our studies elucidate an important mechanism and pathway for the degradation of SnoN and more importantly, reveal a novel role of the APC in the regulation of TGFß signaling.

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INTRODUCTION

Smad proteins mediate transforming growth factor-ß (TGFß) signaling to regulate cell growth and differentiation. SnoN is an important negative regulator of TGFß signaling that functions to maintain the repressed state of TGFß target genes in the absence of ligand (Stroschein et al. 1999; Sun et al. 1999). Upon TGFß stimulation, Smad3 and Smad2 translocate into the nucleus and induce a rapid degradation of SnoN, allowing activation of TGFß target genes (Heldin et al. 1997; Massague et al. 2000; Chacko et al. 2001; Wu et al. 2001). We examined the mechanism of TGFß- and Smad-dependent turnover of SnoN and have identified key components of the ubiquitin machinery involved in the process. Our studies suggest that the anaphase promoting complex (APC) is involved in the Smad3-induced ubiquitination and degradation of SnoN. Since the transforming activity of SnoN is closely correlated with its high steady-state level, the elucidation of mechanisms regulating the intracellular level of SnoN may provide critical insights into the cause of oncogenic transformation by SnoN.

BODY

SnoN is an important negative regulator of TGFß signaling that functions to maintain the repressed state of TGFß target genes in the absence of ligand and in the negative feedback control of TGFß signaling (Stroschein et al. 1999; Sun et al. 1999). Degradation of SnoN is an essential initial step for the activation of TGFß signaling (Stroschein et al. 1999). In the last year, I have shown that TGFß-induced degradation of SnoN requires direct interaction of SnoN with Smad2 or Smad3, is dependent on the ubiquitin-dependent proteasome and can be mediated by the anaphase promoting complex (Stroschein et al. 2001, see appendix for paper submitted). At least three regions in SnoN are required for its ubiquitination and degradation in response to TGFß1. These include a Smad2/3 binding domain, lysine residues that are ubiquitinated and the D box that is targeted by the CDH1-APC. Mutation of any of the three regions completely blocked TGFß- and Smad3-induced ubiquitination and degradation of SnoN. In the absence of stimuli, SnoN can interact with APC-CDH1 and undergo a low level of constitutive ubiquitination. This may function to maintain the low steady-state level of SnoN in mammalian cells. However, since the D box in SnoN is not a perfect D-box, its affinity for the APC-CDH1 is low, and this interaction needs to be markedly enhanced by Smad3 or Smad2 to cause efficient ubiquitination of SnoN. These results are consistent with the model that upon activation of TGFß signaling and subsequent nuclear accumulation of the R-Smads, Smad2 or Smad3 interacts with both the APC and SnoN, resulting in recruitment of the APC to SnoN. In the presence of the UbcH5 family of E2 conjugating enzymes, the poly-ubiquitin chain is then added to the critical lysines 440, 446 or 449 of SnoN, leading to its degradation. Consistent with this model, we found

that purified APC, together with recombinant E1 and UbcH5, can induce poly-ubiquitination of SnoN in an in vitro reconstituted assay in a R-Smad-dependent manner.

Our work has provided the first evidence linking the APC to TGFß signaling. In this case, Smad2 and Smad3 are serving as substrate-specific targeting subunits of the APC. It is unclear whether the APC is responsible for the degradation of other TGFß signaling molecules or whether TGFß induces ubiquitination and degradation of other APC substrates.

Since Smad2 and Smad3 have been shown to function in concert with the Hect-domain ubiquitin ligase, Smurf2 (Bonni et al. and data not shown), and with the APC to induce degradation of SnoN, at least two ubiquitination pathways can be activated by TGFß to induce degradation of SnoN. These two pathways may function either synergistically or differentially in a tissue type- or developmental stage-specific manner to regulate SnoN level and TGFß responses. The involvement of multiple ubiquitination pathways has also been demonstrated for the degradation of ß-catenin (Polakis 2001). Future experiments will investigate the contribution of each pathway in regulation of SnoN expression.

KEY RESEARCH ACCOMPLISHMENTS

Task 1. To analyze the role of Smad3 in SnoN degradation

- Demonstrated that a direct interaction between SnoN and Smad2 or Smad3 is required for SnoN degradation.

Task 2. To further characterize the mechanism of SnoN degradation

- Demonstrated that Smad3 and TGFβ induced degradation of SnoN is dependent on the ubiquitin dependent proteasome.
- Identified the lysine residues on SnoN which are ubiquitinated and are required for SnoN degradation.
- Identified the Anaphase Promoting Complex (APC) as an E3 ubiquitin ligase for SnoN.
- Show that TGFβ signaling induces Smad3 to recruit SnoN to the APC for SnoN ubiquitination and subsequent degradation.
- Demonstrate that SnoN degradation is required for TGFβ signaling to induce growth arrest of lymphoid cells.

REPORTABLE OUTCOMES

Manuscript submitted to Genes and Development, May 2001 (see appendix)

Stroschein, S.L., Bonni, S., Wrana, J.L. and Luo, K. 2001. Smad3 recruits the Anaphase Promoting Complex for ubiquitination and degradation of SnoN. *Genes & Dev.* Submitted

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CONCLUSIONS

I have demonstrated that TGFβ signaling induces SnoN degradation in a Smad2/Smad3 dependent manner by inducing recruitment of SnoN to the APC by Smad2/Smad3. Recruitment of SnoN to the APC leads to ubiquitination and subsequent degradation of SnoN. The reduction of SnoN levels through degradation frees the Smads from negative regulation allowing full activation of TGFβ signaling and its function as a tumor suppressor.

Deregulation of protein degradation can lead to tumorigenesis through misregulated levels of critical oncogene products or tumor suppressor proteins. The transforming oncoprotein v-Jun, a truncated version of c-Jun, lacks a 27 amino acid domain required for Jun degradation (Treier et al. 1994). The lack of degradation results in an increased cellular level of v-Jun, and this may contribute to its transforming ability. The G1 CDK inhibitor $p27^{Kip1}$ is found in decreased levels in aggressive colorectal cancers due to increased degradation of p27 (Loda et al. 1997). SnoN was originally defined as an oncogene because high levels of wildtype SnoN lead to transformation of chicken and quail embryo fibroblasts (Boyer et al. 1993). High levels of SnoN are also detected in lymphomas and in carcinoma cells of the stomach, thyroid and lung (Nomura et al. 1989). It is still not clear how the expression level of SnoN is elevated in cancer cells. My current study has revealed the cellular mechanism and pathways for the regulation of SnoN degradation and provided a potential mechanism for the induction of oncogenic potential of SnoN.

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Smad3 recruits the anaphase promoting complex for ubiquitination and degradation of SnoN

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Abstract

Smad proteins mediate transforming growth factor-β (TGFβ) signaling to regulate cell growth and differentiation. SnoN is an important negative regulator of TGFβ signaling that functions to maintain the repressed state of TGFβ target genes in the absence of ligand. Upon TGFβ stimulation, Smad3 and Smad2 translocate into the nucleus and induce a rapid degradation of SnoN, allowing activation of TGFβ target genes. Here we show that Smad2- or Smad3-induced degradation of SnoN requires the ubiquitin-dependent proteasome and can be mediated by the anaphase promoting complex (APC) and the UbcH5 family of ubiquitin conjugating enzymes. Smad3 and to a lesser extent, Smad2, interact with both the APC and SnoN, resulting in the recruitment of the APC to SnoN and subsequent ubiquitination of SnoN in a destruction box-dependent manner. In addition to the destruction box, efficient ubiquitination and degradation of SnoN also requires the Smad3 binding site in SnoN as well as key lysine residues necessary for ubiquitin attachment. Mutation of either the Smad3 binding site or lysine residues results in stabilization of SnoN and in enhanced antagonism of TGFβ signaling. Our studies elucidate an important mechanism and pathway for the degradation of SnoN and more importantly, reveal a novel role of the APC in the regulation of TGFβ signaling.

Introduction

Smad proteins are critical components of the TGFβ signaling pathways. In the absence of TGFβ, the two highly homologous receptor-associated Smads, Smad2 and Smad3, are distributed mostly in the cytoplasm. Upon ligand binding, the activated type I TGFβ receptor serine/threonine kinase (TβRI) phosphorylates these Smad proteins, allowing them to translocate into the nucleus and form heteromeric complexes with the common-mediator Smad4 (Heldin et al. 1997; Massague et al. 2000; Chacko et al. 2001; Wu et al. 2001). In the nucleus, the Smad complexes interact with various cellular partners and participate in diverse downstream activities. The Smads can bind to the TGFβ-responsive promoter DNA either directly through the N-terminal Mad homology-1 (MH1) domain (Massague et al. 2000) or in conjunction with other sequence-specific DNA binding proteins such as the FAST and Milk family of proteins (Chen et al. 1996; Chen et al. 1997; Liu et al. 1997a; Labbe et al. 1998; Zhou et al. 1998b; Germain et al. 2000). Through the C-terminal MH2 domains, Smad proteins interact with general or promoter-specific transcriptional activators to activate transcription of various TGFβ target genes (Massague and Wotton 2000). They may also associate with transcriptional co-repressors such as TGIF (Wotton et al. 1999), Ski (Akiyoshi et al. 1999; Luo et al. 1999; Sun et al. 1999a), SnoN (Stroschein et al. 1999b; Sun et al. 1999b) and SNIP1 (Kim et al. 2000). Some activated Smads can also be targeted for degradation by the ubiquitin-dependent proteasome (Lo and Massague 1999; Zhu et al. 1999; Lin et al. 2000; Zhang et al. 2001).

Among the negative regulators of Smad function, SnoN and Ski are two closely related members of the Ski family of nuclear proto-oncoproteins. When overexpressed, they cause oncogenic transformation of chicken and quail embryo fibroblasts as well as muscle

differentiation of quail embryo cells (Colmenares and Stavnezer 1989; Colmenares et al. 1991; Boyer et al. 1993). High levels of Ski or SnoN are detected in many types of human cancer cells (Nomura et al. 1989; Fumagalli et al. 1993). Interestingly, mice lacking one copy of the sno gene were also found to be more susceptible to chemical carcinogens (Shinagawa et al. 2000). Thus, the oncogenic potential of SnoN appears to be related to deregulation of its normal expression levels. Human SnoN is a ubiquitously expressed transcriptional co-repressor of 684 amino acids (Nomura et al. 1989) that interacts with Smad2, Smad3 and Smad4 to antagonize TGFβ signaling (Stroschein et al. 1999b; Sun et al. 1999b). We have shown that SnoN may function to maintain the repressed state of TGFβ target genes in the absence of TGFβ and may also participate in negative feedback control of TGFβ signaling (Stroschein et al. 1999b). Upon TGFβ stimulation, a rapid degradation of SnoN occurs, most likely mediated by Smad3 and to a lesser extent, Smad2. The removal of the inhibitory SnoN may be crucial for activation of TGFβ signaling as this allows the Smads to activate transcription of TGFβ responsive genes.

In this report, we investigated the molecular mechanism by which Smad3 and Smad2 induce degradation of SnoN. Degradation of most intracellular proteins is mediated by the proteasome, which is an essential component of the ATP-dependent proteolytic pathway and is present in the nucleus and cytosol of all eukaryotic cells (Coux et al. 1996). Most proteins degraded by the 26S proteasome require prior poly-ubiquitination. Ubiquitination occurs through a stepwise action of three classes of enzymes (Varshavsky 1997; Hershko and Ciechanover 1998). Initially, ubiquitin is activated by the E1 ubiquitin activating enzyme and transferred to the E2 ubiquitin conjugating (Ubc) enzymes. In the presence of an E3 ubiquitin ligase, the ubiquitin is covalently attached to a particular lysine(s) in the substrate. The cycle

repeats to form a poly-ubiquitin chain, leading to the recognition and degradation of the target protein by the proteasome.

The E3 ubiquitin ligases often interact with the substrates in a regulated manner and play central roles in determining the specificity of ubiquitination. Among the E3s, the Hect-domain containing E3s and Ring-finger containing E3s have been characterized extensively in mammalian cells. The Hect-domain E3s are characterized by the ability to form a thiolester intermediate with the activated ubiquitin before transferring the ubiquitin to the substrates (Scheffner et al. 1993; Huibregtse et al. 1995). One Hect-domain E3 family, the Smurf family, has been shown to interact with the PY motifs present in the N-terminal and linker regions of the Smads and mediates degradation of TGFβ signaling molecules (Zhu et al. 1999; Kavsak et al. 2000; Lin et al. 2000; Bonni et al. 2001; Zhang et al. 2001). For example, Smurf2, through interaction with Smad7, can be recruited to the TGFβ receptors, leading to their degradation (Kavsak et al. 2000). By interacting with Smad2, Smurf2 can also be recruited to SnoN to induce its degradation (Bonni et al. 2001). High levels of Smurf2 have also been shown to degrade Smad1 (Zhang et al. 2001) and Smad2 (Lin et al. 2000) in transfected cells. In contrast to the Hect-domain E3s, the Ring-finger containing E3s do not form a covalent bond with the activated ubiquitin. Many ring-finger containing E3s are part of a large protein complex, such as the anaphase promoting complex (APC) or SCF, that also contain other subunits required for substrate recognition and recruitment and for regulation of the E3 ligase activity. The APC is a cell-cycle regulated ubiquitin-protein ligase that consists of 11 core subunits in mammalian cells (Gmachl et al. 2000), one of which, APC11, is the ring-finger-containing subunit with the E3 ligase activity (Gmachl et al. 2000; Leverson et al. 2000). Two WD-40 proteins, CDC20 and CDH1, can associate with the APC and activate its activity in a substrate-specific and cell cycle-dependent manner (Peters 1999;

Zachariae and Nasmyth 1999). CDC20 interacts with the APC during mitosis and targets proteins containing a destruction box (D box), such as cyclin B and Pds1, for degradation (Fang et al. 1998; Lorca et al. 1998; Shirayama et al. 1999; Kramer et al. 2000; Rudner and Murray 2000). CDH1, present in G1 (Fang et al. 1998) and also in differentiated cells (Gieffers et al. 1999), can recognize the D box or Ken box motifs present in cell-cycle-specific as well as non-cell-cycle proteins and activate the APC for their ubiquitination (Pfleger and Kirschner 2000). Thus, the APC can function in both proliferating and differentiated cells and may regulate the degradation of both cell-cycle and non-cell-cycle proteins.

In this report, we examined the mechanism of TGFβ- and Smad-dependent turnover of SnoN and have identified key components of the ubiquitin machinery involved in the process. Our studies suggest that the anaphase promoting complex (APC) is involved in the Smad3-induced ubiquitination and degradation of SnoN. Since the transforming activity of SnoN is closely correlated with its high steady-state level, the elucidation of mechanisms regulating the intracellular level of SnoN may provide critical insights into the cause of oncogenic transformation by SnoN.

Results:

TGFβ- and Smad3-induced degradation of SnoN is mediated by the Ubiquitin-dependent proteasome.

We have previously shown that TGFβ induces a rapid degradation of SnoN (Stroschein et al. 1999b). This activity appears to be mediated by Smad3 and to a slightly lesser extent, Smad2, because overexpression of Smad2 or Smad3 resulted in degradation of SnoN even in the absence of TGFβ signaling (Figure 1A). It is known that overexpressed Smad2 and Smad3 can partially bypass the requirement for ligand and localize in the nucleus, oligomerize and activate downstream events (Wu et al. 1997; Zhang et al. 1997). Activation of TGFβ signaling can further enhance these events. This property of the Smads allows us to directly investigate the effects of Smad proteins on degradation of SnoN.

To investigate the mechanism responsible for Smad3-induced degradation of SnoN, we first employed inhibitors of various intracellular degradation machineries in pulse-chase assays. ALLM, an inhibitor of Ca^{2+}-activated proteases (calpains), had no effect on Smad3-induced degradation of SnoN (Fig. 1B). In contrast, lactacystin, an inhibitor specific for the proteasome, and MG132, a proteasome and calpain inhibitor, blocked SnoN degradation (Figure 1B). Furthermore, treatment of Hep3B cells with lactacystin also prevented TGFβ-induced degradation of endogenous SnoN proteins (Figure 1C), suggesting that TGFβ-induced, Smad3-dependent degradation of SnoN is mediated by the proteasome.

Since most proteins degraded by the proteasome require ubiquitination (Hershko and Ciechanover 1998), we asked whether Smad2 and Smad3 induce ubiquitination of SnoN. SnoN was isolated from transfected cells by immunoprecipitation with an anti-SnoN antiserum and

detected by immunoblotting with anti-ubiquitin. In the absence of Smad proteins, SnoN exhibited a low level of poly-ubiquitination (lane 4, Figure 1D). Co-expression of SnoN with Smad3 or Smad2, but not with Smad4, resulted in a marked increase in the poly-ubiquitination of SnoN (lanes 5-7, Figure 1D). This is consistent with the observation that Smad3 and Smad2, but not Smad4, induced degradation of SnoN (Figure 1A).

A direct interaction between Smad3 and SnoN is necessary but not sufficient for the degradation of SnoN

The ubiquitination and degradation of SnoN are mediated by the MH2 domains of Smad2 and Smad3 (Smad2C and Smad3C) (Figure 1A and 1E), but not by the amino-termini and linker regions (Smad3N and Smad2NL) that do not interact with SnoN. To further define the region in Smad2 and Smad3 required for binding and degradation of SnoN, carboxyl-terminal deletions of Smad3 and chimeras between Smad1 and Smad2 were tested in binding assays (Figures 2A and 2B). All Smad3 deletion mutants bound SnoN, albeit weakly (Figure 2A, lanes 5-8). This suggests that the SnoN binding site lies between amino acid residues 230 and 289. The weak binding is most likely due to the inherent instability of these deletions compared to the wildtype Smad3 (Figure 2A and data not shown). To map the SnoN binding site in Smad2, Smad1/Smad2 chimeras were employed. Since Smad1 does not bind to SnoN (lane 3, Figure 2B), any binding to SnoN is contributed by the Smad2 portion of the molecule. Chimeras containing amino acids 279-319 from Smad2 (equivalent to residues 237-276 in Smad3) bound to SnoN (lanes 4 and 8-9, Figure 2B), while those containing the equivalent region from Smad1 did not (lanes 5-7, Figure 2B). Thus, the equivalent region in Smad3 (residues 230-289) and in Smad2 (residues 279-319) mediates binding to SnoN.

We next tested whether this region is necessary and sufficient for degradation of SnoN. Since deletion of sequences in the MH2 domains of the Smads may affect the folding and stability of the proteins, we employed two Smad3/Smad1 chimeras in this experiment, one (S3/S1/S3) with the SnoN binding domain of Smad3 replaced with the Smad1 sequence (Smad3 residues 1-236 and 277-425, Smad1 residues 276-317) and another (S1/S3/S1) with only the SnoN binding domain of Smad3 (Smad1 residues 1-275 and 318-465, Smad3 residues 237-276). S3/S1/S3 was unable to bind SnoN and did not induce ubiquitination (Figure 2C and data not shown) or degradation of SnoN (Figure 2C). S1/S3/S1, on the other hand, bound SnoN. However, this chimera was unable to induce degradation of SnoN to the same extent as wt Smad3 (Figure 2C), suggesting that amino acid residues 237-276 in Smad3 are sufficient for binding to SnoN, but not for SnoN degradation. Interaction of Smad3 with other cellular factors, presumably the ubiquitination machinery, is also required for efficient degradation of SnoN (Figure 6A). In this sense, Smad3 or Smad2 serves as the targeting subunit of an E3 ligase complex.

Domains in SnoN required for ubiquitination and degradation

At least two determinants are required for a protein to be ubiquitinated and degraded by the proteasome: recognition site(s) for the targeting subunit(s) of an E3 ubiquitin ligase and a lysine residue(s) that serves as an ubiquitin attachment site (Hershko and Ciechanover 1998). Deletion mutants of SnoN were generated to map these two determinants. Deletion of the first 96 amino acids (SnoN 97-684) disrupted binding to Smad3 ((Stroschein et al. 1999b) and Figure 3B). Within this region, amino acid residues 89-92 appeared to be required for binding to Smad3 as substitutions of these residues with alanine (SnoN 89-92A) disrupted binding to Smad2 or Smad3 (Figure 3B, third panel from the top and J. He and K. Luo manuscript in preparation).

Neither the deletion mutant nor the point mutant was ubiquitinated (Figure 3B) or degraded in response to Smad3 co-expression (Figure 3C). Thus, interaction with Smad2 or Smad3 is required for ubiquitination and degradation of SnoN. When expressed in the TGFβ-responsive Hep3B cells, SnoN 89-92A also failed to be degraded in response to TGFβ (Figure3D), again confirming that TGFβ-induced degradation of SnoN is mediated by interaction with Smad2 and Smad3.

We have previously shown that a mutant SnoN containing only the amino-terminal 366 amino acids (SnoN 1-366) can not be degraded by the co-expression of Smad3 (Stroschein et al. 1999b). Since this mutant still binds Smad3, an additional element required for SnoN degradation must be present in the carboxyl half of SnoN between residues 367-684. In the ubiquitination assay, this mutant failed to be ubiquitinated in the presence of Smad3 (data not shown). Thus, the lysine residues required for ubiquitination may be located in the carboxyl half of SnoN and are missing in SnoN 1-366. Serial deletions from the carboxyl terminus of SnoN were made, and these deletion mutants were tested for their ability to be degraded by Smad3 co-expression. Since some SnoN truncations have similar molecular mass with Smad3 and therefore are difficult to resolve on an SDS-PAGE gel, Smad2C was used in the pulse chase assay to induce degradation of SnoN. Deletions up to residue 459 (SnoN 1-459) had no effect on Smad2C-induced degradation of SnoN (Figure 4B and data not shown). This mutant was degraded by the co-expression of Smad2C with kinetics similar to that of wild-type SnoN (Figure 4B). Thus, the lysine residue(s) must reside between residues 367-459.

There are eight lysine residues within this region (Figure 4A). These eight residues were mutated individually or in various combinations to arginine or alanine, and the resulting mutants tested in ubiquitination and degradation assays. Alteration of the first five lysines (Lys 383, 407,

423, 427, 432), SnoN K1-5R, had no effect on Smad3-induced ubiquitination (Figure 4C). In contrast, mutation of the last three lysines, Lys 440, 446 and 449, either alone (SnoN K6R, K7-8R) or in combination with other lysine residues (SnoN K6-8R, K1-6R) abolished Smad3-induced ubiquitination (Figure 4C). Consistent with this, mutant SnoN lacking any of the three lysine residues can not be degraded by TGFβ when transfected into Hep3B cells (Figure 4D). Thus lysines 440, 446 and 449 are important for both ubiquitination and degradation of SnoN.

The UbcH5 family of E2 ubiquitin conjugating enzymes may be required for Smad3-induced SnoN degradation

To identify the cellular machinery responsible for SnoN ubiquitination and degradation, various dominant negative E2 ubiquitin conjugating enzymes were tested for their ability to block Smad3-induced degradation of SnoN in pulse-chase assays. These mutant E2 enzymes contain catalytic site mutations and have been shown to behave in a dominant negative manner (Gonen et al. 1999). Co-transfection of dominant negative mutants of the closely related UbcH5a, UbcH5b, or UbcH5c inhibited Smad3-induced SnoN degradation (Figure 5A). In contrast, dominant negative UbcH6, UbcH7, UbcH8, UbcH9 and UbcH10 had no effect on the half-life of SnoN in the presence of Smad3 (Figure 5A), even though all these mutant E2s were expressed well in transfected cells (data not shown). This suggests that the UbcH5 family of E2 enzymes may mediate Smad3-induced degradation of SnoN. Since the UbcH5 family of E2's has been shown to function in concert with both the Hect-domain E3 ubiquitin ligase E6-AP and the anaphase promoting complex or cyclosome (APC) that targets the D box motif, ubiquitination of SnoN may involve members of these ubiquitin ligase families (Scheffner et al. 1994; Jensen et al. 1995; Rolfe et al. 1995; Gmachl et al. 2000; Leverson et al. 2000).

Smad3-induced degradation of SnoN is mediated by a D box-dependent ubiquitination pathway

We have recently shown that Smurf2, a Hect-domain E3 ubiquitin ligase, can be recruited to SnoN through interaction with Smad2 or Smad3 in a TGFβ-dependent manner to mediate degradation of SnoN (Bonni et al. 2001 and data not shown). This interaction requires the PY motif in the MH1/linker regions of Smad2 or Smad3 (Bonni et al. 2001 and data not shown). However, Smurf2 may not be the only E3 ligase responsible for Smad3-induced degradation of SnoN, since a mutant Smad3 lacking the PY motif (Smad3ΔPY) still induced ubiquitination and degradation of SnoN (Figure 5B), albeit less efficiently than the wt Smad3, even though this Smad3ΔPY was unable to bind to Smurf2 (data not shown). Furthermore, the MH2 domains of Smad3 and Smad2, which do not contain the PY motifs, can induce ubiquitination and degradation of SnoN (Figure 1A). Thus, an additional Smurf2-independent ubiquitination pathway can be recruited by Smad3 and Smad2 to induce degradation of SnoN. Activation of both pathways may be required for maximal degradation of SnoN.

A D box motif was found between residues 164-172 that shows a consensus of RxxLxxxxN (Figure 5C and Marc W. Kirschner, personnel communication). This motif, targeted by the APC, is usually found in cyclins and other cell cycle-regulated proteins and mediates degradation of these proteins (Glotzer et al. 1991; Peters 1999). To determine whether the D box is required for Smad3-induced degradation of SnoN, two critical residues in the D box were mutated, and the resulting SnoN mutant was tested in the pulse-chase assay. As shown in Figure 5C, mutation of the D box (mDbox) prevented Smad3-induced ubiquitination and degradation of SnoN. Thus, the D box is essential for Smad3-induced degradation of SnoN, and a ubiquitin

ligase complex that targets the D box, possibly the APC, is required for Smad3-induced degradation of SnoN.

Smad3 and Smad2 interact with the APC to induce ubiquitination of SnoN

To investigate whether the APC can be recruited by Smad3 and Smad2 to induce degradation of SnoN, we first examined the ability of the Smads to interact with the APC components in the nucleus by a co-immunoprecipitation assay. In nuclear extracts prepared from 293T cells transfected with Flag-Smad3, Flag-Smad3 was found to complex with endogenous Cdc27 and Cdc16, two important components of the APC (Peters 1999; Zachariae and Nasmyth 1999), and this interaction was mediated by the C-terminal MH2 domain of Smad3 (Figure 6A). Activation of TGFβ signaling can further enhance this interaction (Figure 6C). The APC-binding site in the Smad3 MH2 domain is outside the SnoN-binding region because the S1/S3/S1 chimera, which is sufficient for binding to SnoN, failed to interact with the APC (lane 8, Figure 6A), while the S3/S1/S3 did not bind to SnoN, but associated with the APC (lane 7). Since neither chimera induced efficient degradation of SnoN, binding of Smad3 to both SnoN and the APC may be necessary for ubiquitination of SnoN. In addition to Smad3, Smad2 but not Smad4 also formed a complex with endogenous APC (Figure 6A), albeit to a lesser extent, consistent with the fact that only Smad3 and Smad2, but not Smad4, induce degradation of SnoN.

CDH1, the targeting subunit of the APC, recognizes the D box or Ken box motifs in the substrate proteins and activates the APC for the ubiquitination of these substrates (Pfleger and Kirschner 2000). To examine whether CDH1 can interact with SnoN through its D box, cell extract was prepared from 293T cells co-transfected with Flag-SnoN and HA-CDH1. SnoN was found to interact with CDH1 (lane 4, Figure 6B), and this interaction was mediated by the N-terminal half of SnoN (lane 5) and depended on the D-box of SnoN since a mutant SnoN lacking

the D-box did not bind CDH1 (lane 7, Figure 6B). In contrast, neither Smad3 nor Smad4 associated with CDH1 in the same experiment (Figure 6B). Thus, in the absence of TGFβ stimulation, SnoN could be targeted by the APC, albeit with a low affinity, through CDH1, resulting in the low level of constitutive ubiquitination of SnoN (lane 4, Figure 1D). Consistent with this, a low level of interaction between SnoN and endogenous Cdc27 and Cdc16 was detected even in the absence of TGFβ or Smad3 (lane 1, top panel, Figure 6C).

We next examined whether Smad3, following activation by TβRI, could recruit additional APC to SnoN to enhance the APC-CDH1-SnoN interaction. Since expression of Smad3 induces degradation of SnoN, which precludes the analysis of SnoN, a proteasome inhibitor, MG132, was used to pre-treat the cells to prevent degradation of SnoN. In order to detect the dependency of Smad3 activity on activation of TGFβ signaling, we reduced the expression level of Smad3 so that its nuclear localization and oligomerization activity is dependent on the expression of a constitutively active TβRI. In the absence of the active TβRI, Smad3 associated with the APC components and SnoN in the nucleus (lane 3, Figure 6C) as shown before, probably because a small portion of transfected Smad3 is located in the nucleus. However, this low level of interaction did not result in any significant recruitment of the APC to SnoN (lane 3, top panel). In contrast, in nuclear extract prepared from cells expressing the constitutively active TβRI, increased associations between Smad3 and the APC (lane 4, second panel from the top, Figure 6C) and between Smad3 and SnoN (data not shown) were detected. As a result, a marked increase in the interaction of SnoN with the APC components was observed (lane 4, top panel, Figure 6C). This increased interaction of SnoN with the APC was mediated by Smad3 because the interaction of the APC with the mutant SnoN (SnoN 89-92A) deficient in binding to Smad3 was not enhanced by Smad3 (lanes 7-9, Figure 6C), and there was no increase in the APC and

SnoN interaction in cells expressing only SnoN and the constitutively active TBRI (lane 2, Figure 6C). Similarly, Smad2 can also recruit the APC to SnoN in response to TGFβ signaling, albeit with a lower efficiency (lanes 5-6, Figure 6C). Thus, activation of TGFβ signaling could result in an increased interaction of Smad3 and to a lesser extent, Smad2, with the APC and subsequent recruitment of the APC to SnoN, leading to efficient ubiquitination and degradation of SnoN. When Smads were overexpressed, they were able to recruit the APC to SnoN even in the absence of ligand (data not shown), consistent with the observation that overexpression of Smad2 or Smad3 in 293T cells can induce ubiquitination and degradation of SnoN. In TGFβ-responsive Ba/F3 cells, TGFβ also induced the formation of an endogenous Smad3/SnoN/APC complex (Figure 6D), again supporting the model that TGFβ stimulation results in the recruitment of the APC to SnoN by Smad3.

To confirm that the APC can induce ubiquitination of SnoN in a Smad3-dependent manner, an in vitro reconstituted ubiquitination assay was performed with the immunoprecipitated APC from 293T cells and recombinant E1, UbcH5b and UbcH5c (E2), and GST-ubiquitin. Flag-SnoN was purified from transfected 293T cells by immunoprecipitation with anti-Flag agarose, eluted with the Flag peptide and incubated with the above enzymes in vitro in the absence or presence of Smad3. Poly-ubiquitinated species of SnoN were then detected by immunoblotting with the anti-Flag antibody. As shown in Figure 6E, in the absence of the Smads, a low level of poly-ubiquitination of SnoN was detected in vitro, dependent on the presence of E1 and E2 (lane 2). This low level of constitutive poly-ubiquitination of SnoN may be due to the presence of a low level of endogenous E3 ligase co-purified with Flag-SnoN. The addition of purified APC only marginally increased this constitutive ubiquitination (lane 3, Figure 6E). However, an exogenously added CDH1 significantly enhanced ubiquitination of SnoN (data

not shown), suggesting that the APC-CDH1 can mediate ubiquitination of SnoN. In a similar reaction, Smad2 or Smad3 themselves were not ubiquitinated by APC (lanes 14 and 15, Figure 6E), consistent with the observation that CDH1, which is required for the targeting and activation of the APC, did not interact with Smad3 (lane 3, Figure 6B). Interestingly, addition of Smad3 and APC in the reaction markedly enhanced the poly-ubiquitination of SnoN (lanes 3 and 4, Figure 6E). This enhancement required the direct interaction between Smad3 and SnoN, because ubiquitination of the SnoN 97-684 mutant that can not bind to Smad3 was not affected by the presence of Smad3 (lanes 8 and 9, Figure 6E), and was dependent on the D box, as the ubiquitination of the D box mutant (mDbox) was not enhanced by Smad3 (lanes 10 and 11, Figure 6E). Consistent with previous observations, SnoN 1-366 that lacks the critical lysine residues required for ubiquitin attachment was not ubiquitinated by APC either in the absence or presence of Smad3 (lanes 6 and 7, Figure 6E). Although a low level of constitutive ubiquitination of the K6-8R mutant was observed in the presence of the APC, presumably through other neighboring lysine residues, this ubiquitination was not enhanced by Smad3 (lanes 12 and 13, Figure 6E), again supporting the model that Lysines 440, 446 and 449 are required for TGFβ- and Smad3-specific ubiquitination of SnoN. Similar to Smad3, Smad2 also induced ubiquitination of SnoN (lane 5, Figure 6E). Thus, Smad2 and Smad3 can recruit the APC to SnoN to induce ubiquitination and degradation of SnoN.

Stabilization of SnoN enhances its ability to block TGFβ signaling

If mutation of the lysine ubiquitin attachment sites stabilizes the SnoN protein, these mutants should be more potent than wt SnoN in antagonizing TGFβ signaling. To test this hypothesis, wt SnoN or the SnoN K6-8R mutant were introduced stably into the Ba/F3 pro-B cells by retroviral infection, and the pools of infected cells were examined for their ability to

respond to TGFβ-induced growth inhibition. As shown previously, overexpression of wt SnoN attenuated the ability of cells to undergo TGFβ-induced cell cycle arrest only moderately ((Stroschein et al. 1999b), Figure 7B), probably because this molecule is degraded following TGFβ stimulation and therefore can not be maintained at a very high level in these cells (lane 3, Figure 7A). In contrast, SnoN 1-366, which can not be degraded, greatly impaired TGFβ-induced growth inhibitory response ((Stroschein et al. 1999b), Figures 7A and 7B). Mutation of the three lysine residues (SnoN K6-8R) impaired SnoN degradation in response to TGFβ (compare lane 3 to lane 5, Figure 7A) and enhanced the ability of SnoN to attenuate cell cycle arrest moderately, but reproducibly (Figure 7B). The fact that SnoN K6-8R did not block TGFβ signaling to the same extent as SnoN 1-366 may be due to its lower expression level (Figure 7A). Thus, stabilization of SnoN through mutation of the ubiquitin attachment sites can further enhance attenuation of TGFβ-induced growth inhibition by SnoN. This again supports that notion that intracellular degradation of SnoN plays an important role in the regulation of TGFβ signaling.

Discussion

SnoN is an important negative regulator of TGFβ signaling that functions to maintain the repressed state of TGFβ target genes in the absence of ligand and in the negative feedback control of TGFβ signaling. Degradation of SnoN is an essential initial step for the activation of TGFβ signaling (Stroschein et al. 1999b). In this report, we have shown that TGFβ-induced degradation of SnoN requires direct interaction of SnoN with Smad2 or Smad3, is dependent on the ubiquitin-dependent proteasome and can be mediated by the anaphase promoting complex. At least three regions in SnoN are required for its ubiquitination and degradation in response to TGFβ1 (Figure 8). These include a Smad2/3 binding domain, lysine residues that are ubiquitinated and the D box that is targeted by the CDH1-APC. Mutation of any of the three regions completely blocked TGFβ- and Smad3-induced ubiquitination and degradation of SnoN. In the absence of stimuli, SnoN can interact with APC-CDH1 and undergo a low level of constitutive ubiquitination. This may function to maintain the low steady-state level of SnoN in mammalian cells (Figure 8). However, since the D box in SnoN is not a perfect D-box, its affinity for the APC-CDH1 is low, and this interaction needs to be markedly enhanced by Smad3 or Smad2 to cause efficient ubiquitination of SnoN. These results are consistent with the model (Figure 8) that upon activation of TGFβ signaling and subsequent nuclear accumulation of the R-Smads, Smad2 or Smad3 interacts with both the APC and SnoN, resulting in recruitment of the APC to SnoN. In the presence of the UbcH5 family of E2 conjugating enzymes, the poly-ubiquitin chain is then added to the critical lysines 440, 446 or 449 of SnoN, leading to its degradation. Consistent with this model, we found that purified APC, together with recombinant E1 and UbcH5, can induce poly-ubiquitination of SnoN in an in vitro reconstituted assay in a R-Smad-dependent manner.

The APC is a multi-subunit ubiquitin-protein ligase complex that targets mitotic cyclins and other proteins functioning in late mitosis for degradation, thereby regulating the initiation of anaphase and exit from mitosis (Zachariae and Nasmyth 1999). In addition to these mitosis-related proteins, APC may also degrade proteins that are involved in other important cellular functions in a D box- or KEN box-dependent manner. APC-CDH1 has been shown to induce ubiquitination and degradation of CDC6, a protein critical for the initiation of DNA replication, in a cell cycle-dependent manner (Petersen et al. 2000). Here we provide the first evidence linking the APC to TGFβ signaling. In this case, Smad2 and Smad3 are serving as substrate-specific targeting subunits of the APC. It is unclear whether the APC is responsible for the degradation of other TGFβ signaling molecules or whether TGFβ induces ubiquitination and degradation of other APC substrates. At least in our in vitro reconstituted assays, Smad3 was not ubiquitinated by the APC.

This ability of Smad2 or Smad3 to induce degradation of SnoN is independent of their ability to heterodimerize with Smad4 because a mutant Smad3 defective in homo- and hetero-oligomerization induced SnoN ubiquitination as efficiently as wildtype Smad3 (data not shown). Thus, the population of Smad3 responsible for SnoN degradation may differ from those that complex with Smad4 to regulate transcriptional activation. This is consistent with the observation that Smad3 is present in many different protein complexes of various sizes before and after TGFβ signaling (Jayaraman and Massague 2000). Interestingly, although Smad3 can interact with the APC component to induce degradation of SnoN, its own stability was not affected by the APC (data not shown). Since Smad2 and Smad3 have been shown to function in concert with the Hect-domain ubiquitin ligase, Smurf2 (Bonni et al. and data not shown), and with the APC to induce degradation of SnoN, at least two ubiquitination pathways can be

activated by TGFβ to induce degradation of SnoN. The observation that Smad3ΔPY, deficient in the recruitment of Smurf2, was less efficient than wt Smad3, but nevertheless was capable of mediating the degradation of SnoN (Figure 5B) suggests that both the Smurf2 pathway and the APC pathway are required for maximal degradation of SnoN by the Smads. These two pathways may function either synergistically or differentially in a tissue type- or developmental stage-specific manner to regulate SnoN level and TGFβ responses. The involvement of multiple ubiquitination pathways has also been demonstrated for the degradation of β-catenin (Polakis 2001). Future experiments will investigate the contribution of each pathway in regulation of SnoN expression.

Our results using the dominant negative mutants of various E2 enzymes suggest the involvement of the UbcH5 family of enzymes in TGFβ-induced degradation of SnoN. UbcH5 belongs to an evolutionarily conserved subfamily of E2s that are highly homologous to yeast UBC4/UBC5 (Scheffner et al. 1994; Jensen et al. 1995; Rolfe et al. 1995). This family of ubiquitin conjugating enzymes has been shown to function in concert with the HECT-domain E3 ligases as well as the APC in vitro. In reconstituted ubiquitination assays, UbcH5b (hUBC4) can complex with the HECT-domain ligase, E6-AP, to stimulate ubiquitination of p53 (Scheffner et al. 1994; Jensen et al. 1995; Rolfe et al. 1995) or together with APC11, the RING-finger containing subunit of APC, to induce poly-ubiquitination of cyclin B or securin (Gmachl et al. 2000; Leverson et al. 2000). These functional capacities of UbcH5 are consistent with our findings that both the APC and Smurf2 may be involved in the degradation of SnoN. In addition to SnoN, UbcH5b and UbcH5c have been found to mediate degradation of Smad2 in an activation-dependent manner (Lo and Massague 1999). It is not clear which E3 ligase is involved in that process. Another E2, UbcH10 (E2-C), has also been implicated in APC-mediated

ubiquitination (Aristarkhov et al. 1996; Townsley et al. 1997), but a dominant negative form of this E2 had no effect on Smad3-induced degradation of SnoN (Figure 6A). The three members of UbcH5, UbcH5a, UbcH5b and UbcH5c, are highly related (88% identity between UbcH5a and UbcH5b or UbcH5c and 97% identity between UbcH5b and UbcH5c). It is not clear whether these proteins display any functional specificity in their ability to mediate ubiquitination of SnoN in vivo.

We found that three closely positioned lysine residues in SnoN, Lys 440, 446 and 449, are required for ubiquitination by both the APC (Figure 4C) and Smurf2 (data not shown). Mutation of these residues either individually or in combination greatly impaired the ubiquitination of SnoN and resulted in stabilization of SnoN (Figure 7A) and in enhancement of the ability of SnoN to block TGFβ signaling (Figures 7B). Thus, it is likely that the three lysines, together with the neighboring amino acid residues, form a structure determinant that allows transfer of the ubiquitin moiety to one or more of the three lysines. Alternatively, these lysine residues may function in a cooperative manner to facilitate ubiquitin attachment. SnoN has two alternatively spliced forms, SnoI (399 amino acids) and SnoA (425 amino acids) that share only the first 366 amino acid residues with SnoN (Pearson-White 1993). They interact with Smad2 and Smad3 and contain the D box, but lack the three lysine residues due to truncation of the molecules by alternative splicing. We found that SnoN 1-366 can not be degraded in response to TGFβ stimulation and is more potent in rendering cells resistant to TGFβ-induced growth inhibition. Indeed, SnoI, has been detected in rhabdomyosarcoma tumor (Pearson-White 1993), suggesting that stabilization of SnoN may contribute to the transforming activity of SnoN.

Deregulation of protein degradation can lead to tumorigenesis through misregulated levels of critical oncogene products or tumor suppressor proteins. The transforming oncoprotein v-

Jun, a truncated version of c-Jun, lacks a 27 amino acid domain required for Jun degradation (Treier et al. 1994). The lack of degradation results in an increased cellular level of v-Jun, and this may contribute to its transforming ability. The G1 CDK inhibitor $p27^{Kip1}$ is found in decreased levels in aggressive colorectal (Loda et al. 1997) and breast carcinomas (Catzvaelos et al 1997) due to increased degradation of p27. SnoN was originally defined as an oncogene because high levels of wildtype SnoN lead to transformation of chicken and quail embryo fibroblasts (Boyer et al. 1993). High levels of SnoN are also detected in lymphomas and in carcinoma cells of the stomach, thyroid and lung (Nomura et al. 1989). It is still not clear how the expression level of SnoN is elevated in cancer cells. Our current study has revealed the cellular mechanism and pathways for the regulation of SnoN degradation and provide a potential mechanism for the induction of oncogenic potential of SnoN.

Materials and Methods

Cells, antisera, and constructs

293T and Phoenix-Eco cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The Hep3B human hepatoma cells (ATCC) were maintained in minimal essential medium (MEM) supplemented with 10% FBS. The Ba/F3 pro-B cells were grown in RPMI supplemented with 10% FBS and 10% WEHI cell-conditioned medium as a source of interleukin-3 (Luo and Lodish 1996).

Antisera against ubiquitin (FL-76), SnoN (H-317), Smad3 (FL-425), Cdc27 (C-20) and Cdc16 (K-16) were purchased from Santa Cruz Biotechnology. Antiserum against the Flag peptide was purchased from Sigma. Two polyclonal antibodies against SnoN were described previously (Stroschein et al. 1999b). Smad, SnoN, CDH1 and dominant negative E2 (UbcH6, UbcH7, UbcH8 and UbcH9) mutants were generated by PCR and cloned into pCMV5b. Dominant negative E2 mutations: UbcH5a: C85A; UbcH5b: C85A; UbcH5c: C85A; UbcH6: C131A; UbcH7: C86A; UbcH8: C85A; UbcH9: C93A and UbcH10: C114S. Smad3 ΔPY: lacking residues 180-184.

Transfection and retroviral infection

293T, Phoenix-Eco and Hep3B cells were transiently transfected using lipofectamine-plus (GIBCO Life Technologies). To generate stable Ba/F3 cell lines expressing wt or mutated SnoN, Flag-SnoN in the retroviral vector pMX-IRES-GFP (Liu et al. 1997b) that also expresses the green fluorescent protein (GFP) was used to transfect Phoenix-Eco cells. Forty-eight hours after transfection, $5X10^5$ Ba/F3 cells were co-cultivated with the transfected Phoenix-Eco cells for 24 hours, and the infected cells were selected by cell sorting on the basis of GFP expression.

Immunoprecipitation and western blotting

Flag- and/or HA-tagged Smads and SnoN were isolated from transfected 293T cells by immunoprecipitation with anti-Flag (Sigma) or anti-HA agarose, followed by elution with Flag or HA peptide as described previously (Zhou et al. 1998a; Stroschein et al. 1999a). To detect endogenous APC associated with the Smads, nuclear extracts were prepared from 293T cells transfected with Flag-tagged Smad as described (Lee et al. 1987), and the Flag-tagged protein was isolated by immunoprecipitation with anti-Flag agarose (Sigma), followed by elution with the Flag peptide as described (Zhou et al. 1998a; Stroschein et al. 1999a). Associated Cdc27 and Cdc16 were detected by western blotting with anti-Cdc27 and anti-Cdc16. To detect SnoN and Smad3 associated with the APC, nuclear extracts were prepared from 293T cells transfected with Flag-tagged SnoN and HA-tagged Smad3. The APC was isolated by immunoprecipitation with anti-Cdc27 and anti-Cdc16, and the associated Smad3 and SnoN were detected by western blotting with anti-HA and anti-Flag antibodies. To detect endogenous Smad3 and SnoN associated with the APC, nuclear extracts were prepared from Ba/F3 cells treated for 0 or 20 minutes with TGFβ1. SnoN was isolated by immunoprecipitation with anti-SnoN, and the associated Smad3 and APC were detected by western blotting with anti-Smad3, anti-Cdc27 and anti-Cdc16 antibodies. Immunoprecipitation of endogenous SnoN from Hep3B cells was performed as described (Stroschein et al. 1999b).

To measure TGFβ-induced degradation of SnoN in Ba/F3 cells, $2X10^7$ Ba/F3 cells were serum-starved for 15 hours and treated with TGFβ1 for 0 or 30 minutes. Nuclear extracts were prepared, and Flag-SnoN was detected by western blotting with anti-Flag.

To detect ubiquitination of SnoN in vivo, Flag-SnoN, either singly- or co-transfected with Smads, was isolated from 293T cells by immunoprecipitation with the anti-Flag agarose followed by elution with the Flag peptide. After equalizing the amounts of SnoN in each sample, the levels of SnoN ubiquitination were measured by western blotting with an anti-ubiquitin antiserum (Santa Cruz).

Growth inhibition assay

For growth inhibition assay, $1X10^4$ Ba/F3 cells were incubated with various concentrations of TGFβ1 for 3-4 days. The growth of cells was determined by cell counting and compared with that of unstimulated cells (Luo and Lodish 1996). The assay was set up in triplicate, and standard errors are shown.

Pulse-chase assays

Transfected 293T cells were pulse-labeled for 30 minutes in the presence of ^{35}S-express (0.25mCi/ml, NEN) and chased for various periods of time. SnoN was purified by immunoprecipitation and resolved on an SDS-polyacrylamide gel. For treatments with proteasome inhibitors, transfected cells were pre-treated with 10 μM lactacystin (Calbiochem), 25 μM MG132 (Calbiochem) or 25 μM N-acetyl-Leu-Leu-Methioninal (ALLM) (Sigma) for 45 minutes and then throughout the duration of the pulse and chase.

In vitro ubiquitination assay

Standard ubiquitination assays were carried out in 15 μl of QA (20mM Tris-HCl pH7.7, 100mM KCl, 0.1mM$CaCl_2$, 1mM $MgCl_2$, 1mM DTT) (King et al. 1995; Gmachl et al. 2000) containing 15 μg GST-ubiquitin, 1 μg E1 (Boston Biochem), 1.5 μg E2 (GST-UbcH5b and UbcH5c), 0.6 μl

of an ATP regenerating system and Flag-SnoN and/or Flag-Smad3 or Flag-Smad2 immunopurified from 293T cells. The APC complex was immunoprecipitated from 293T cells with antisera against Cdc27 and Cdc16 (Santa Cruz) and added to the reaction while still immobilized on the Sepharose beads. Reactions were incubated for 30 minutes at 37°C and terminated by the addition of sodium-dodecyl-sulfate (SDS)-sample buffer. Poly-ubiquitinated substrates were detected by western blotting with anti-Flag and anti-SnoN antibodies.

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Figure Legends

Figure 1. Smad3- and TGFβ-induced degradation of SnoN is mediated by the Ubiquitin-dependent proteasome. (**A**) Pulse-chase analysis of SnoN degradation by various Smad proteins. 293T cells co-transfected with HA-SnoN and the indicated Flag-tagged Smads were subjected to pulse-chase assays as described in Materials and Methods. Immunoprecipitation was carried out with anti-Flag to purify Smad-associated SnoN or with anti-HA to purify total cellular HA-SnoN. The half-lives of the two populations were similar. As a control, SnoN from singly transfected cells was isolated by immunoprecipitation with anti-HA. Abbreviations used: S3: Smad3; S3C: MH2 domain of Smad3; S3N: MH1 domain of Smad3; S2: Smad2; S2C: MH2 domain of Smad2; S2NL: MH1 and linker domains of Smad2; S4: Smad4. (**B**) Proteasome inhibitors block Smad3-induced degradation of SnoN. Transfected 293T cells were pre-treated with DMSO (control) or various proteolytic inhibitors for 45 minutes followed by a pulse-chase assay as described in Materials and Methods. Inhibitors were present throughout the duration of the pulse-chase. Quantification of ^{35}S-labeled SnoN was carried out with the Bio-Rad Molecular Imager FX system and is shown below the gels. (**C**) Lactacystin blocks TGFβ-induced degradation of endogenous SnoN. Hep3B cells were pre-treated with DMSO (lanes 1-3) or lactacystin (lanes 4-6) for 4 hours followed by stimulation with 200 pM TGFβ1 for the indicated times. Endogenous SnoN was isolated by immunoprecipitation with an anti-SnoN anti-serum and analyzed by immunoblotting with anti-SnoN. (**D**) Smad2 and Smad3 induce ubiquitination of SnoN. HA-Smads or Flag-SnoN were transfected either alone or together into 293T cells and isolated by immunoprecipitation with either anti-HA (lanes 1-3) or anti-Flag (lanes 4-7). Poly-ubiquitination of SnoN was detected by immunoblotting of the

immunoprecipitates with an anti-ubiquitin antibody (top panel). The amounts of SnoN (middle panel) and Smads present in the immunoprecipitates were shown on the two lower panels. (**E**) The MH2 domain of Smad3 induces SnoN ubiquitination. The experiments were carried out as described in (D).

Figure 2. A direct interaction between Smad3 and SnoN is necessary but not sufficient for degradation of SnoN. (**A**) Residues 230-289 of Smad3 are required for interaction with SnoN. Flag-tagged full-length or truncated Smad3 was co-transfected into 293T cells together with HA-SnoN and isolated by immunoprecipitation with anti-Flag agarose. The immunoprecipitates were subjected to immunoblotting with an anti-HA antibody to detect SnoN that bound to Smad3 (top) or with anti-Flag to control for Smad levels (middle). Cell lysates were blotted with anti-HA to control for SnoN expression (bottom). (**B**) Residues 279-318 of Smad2 are required for interaction with SnoN. Flag-tagged Smad1, Smad2 or Smad1/Smad2 chimeras were co-transfected together with HA-SnoN and analyzed as described in panel A. (**C**) Residues 237-276 of Smad3 are necessary but not sufficient for degradation of SnoN. Left panel: binding assay. Flag-SnoN was transfected either alone or together with HA-tagged Smads and isolated by immunoprecipitation with anti-Flag antibodies followed by western blotting with anti-HA to detect Smads that bound to SnoN (top) or with anti-Flag to detect SnoN (middle). Cell lysates were blotted with anti-HA to control for Smad expression (bottom). Right panel: pulse-chase assay. 293T cells co-transfected with HA-SnoN and Flag-Smad were subjected to pulse-chase assay. S3/S1/S3: Smad3 residues 1-236 and 277-425, and Smad1 residues 276-317. S1/S3/S1: Smad1 residues 1-275 and 318-465, and Smad3 residues 237-276.

Figure 3. Smad2 or Smad3-binding site in SnoN is required for the ubiquitination and degradation of SnoN. (**A**) SnoN mutants. (**B**) Ubiquitination of SnoN requires binding to

Smad2 or Smad3. HA-Smad3 or Flag-SnoN was transfected either individually or together into 293T cells. Flag-SnoN was isolated by immunoprecipitation with anti-Flag (lanes 2-7) antibody followed by western blotting with anti-ubiquitin (top panel), anti-Flag (second panel), or anti-HA (third panel). As a control, HA-Smad3 was isolated from singly transfected cells by immunoprecipitation with anti-HA (lane 1). Total Smad levels were detected by immunoblotting of the cell lysates with anti-HA (bottom panel). (**C**) Pulse-chase assay. 293T cells transfected with HA-SnoN and Flag-Smad3 were subjected to the pulse-chase assay as described in Materials and Methods. SnoN was isolated from the transfected cells by immunoprecipitation with the anti-HA antibody. (**D**) TGFβ-induced degradation of SnoN is mediated by the R-Smads. Hep3B cells were transiently transfected with Flag-tagged wt or mutant SnoN constructs. Cells were treated with TGFβ for 30 min, and SnoN was isolated by immunoprecipitation with anti-Flag agarose and detected by western blotting with anti-Flag antiserum.

Figure 4. Lysines 440, 446, and 449 of SnoN are required for ubiquitination. (**A**) SnoN deletions and point mutations. Lysine residues were mutated to arginine and numbered as follows: 1: K383; 2: K407; 3: K423; 4: K427; 5: K432; 6: K440; 7: K446; and 8: K449. (**B**) Pulse-chase assay of the SnoN deletion mutants. 293T cells were transfected with HA-SnoN and Flag-Smad2C and subjected to the pulse-chase assay. SnoN associated with Smad2C was isolated by immunoprecipitation with the anti-Flag antibody and detected by autoradiography. (**C**) Mutation of lysines 440, 446 and 449 disrupts Smad3-induced ubiquitination of SnoN. Flag-SnoN was co-transfected together with HA-Smad3 and isolated by immunoprecipitation with anti-Flag (lanes 2-15) followed by western blotting with anti-ubiquitin (top panel), anti-Flag (middle panel) or anti-HA antibodies (bottom panel). As a control, HA-Smad3 was isolated from singly transfected cells by immunoprecipitation with anti-HA (lane 1). (**D**) Mutation of lysines

440, 446, and 449 blocks TGFβ-induced degradation of SnoN. Hep3B cells were transiently transfected with the indicated Flag-tagged SnoN constructs. Cells were treated with TGFβ for 30 min, and SnoN was isolated by immunoprecipitation with anti-Flag agarose and detected by western blotting with anti-Flag antiserum.

Figure 5. Ubiquitination pathways and components involved in TGFβ-induced degradation of SnoN. (**A**) The UbcH5 family of E2 ubiquitin conjugating enzymes are required for SnoN degradation. Dominant negative forms of various E2 enzymes were co-transfected into 293T cells together with Smad3 and SnoN. The ability of Smad3 to induce degradation of SnoN was analyzed by pulse-chase assay as described in Materials and Methods. (**B**) Smad3-induced ubiquitination and degradation of SnoN does not require the PY motif of Smad3. Top panel: pulse-chase assay. 293T cells were transfected with SnoN and the Smad3 constructs as indicated and subjected to pulse-chase assays. Bottom panel: ubiquitination assay. HA-Smad3 or Flag-SnoN constructs were transfected either individually or together into 293T cells and isolated by immunoprecipitation with anti-HA (lane 1-2) or anti-Flag (lanes 3-5) antibodies followed by western blotting with anti-ubiquitin (top), anti-Flag (middle) or anti-HA (bottom). (**C**) Smad3-induced ubiquitination and degradation of SnoN requires the D box sequence. Top panel: alignment of various D box sequences. Middle panel: pulse-chase assay. Bottom panel: ubiquitination assay. The experiments were carried out as in (B).

Figure 6. Smad3 and Smad2 interact with the APC to induce ubiquitination of SnoN (**A**) Smad3 and Smad2 interact with the APC components. The indicated Flag-tagged full-length or mutant Smads were transfected into 293T cells and isolated from nuclear extracts by immunoprecipitation with anti-Flag agarose. The immunoprecipitates were subjected to

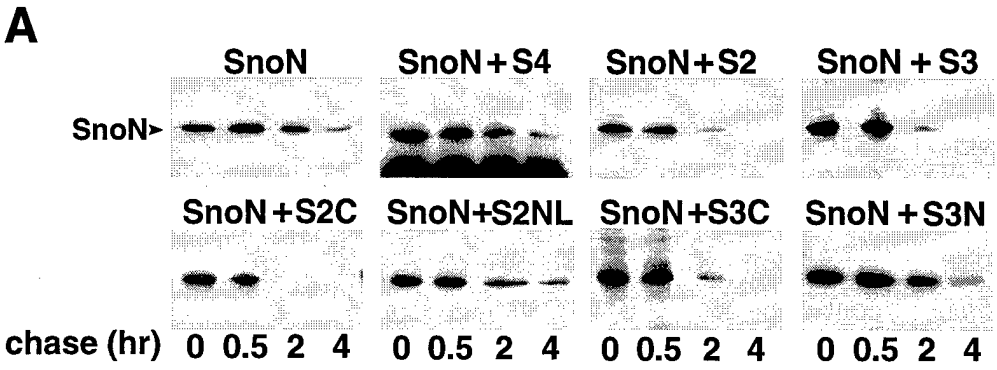
immunoblotting with an anti- Cdc27 antibody (top) or anti- Cdc16 (middle) to detect associated APC components, or with anti-Flag to control for Smad levels (bottom). (**B**) SnoN associates with CDH1 in a D Box-dependent manner. Flag-tagged wt or mutant SnoN or Smads were co-transfected with HA-CDH1 into 293T cells and isolated by immunoprecipitation with anti-Flag agarose. The immunoprecipitates were subjected to immunoblotting with anti-HA to detect CDH1 bound to SnoN (top) and anti-Flag to control for Smad and SnoN levels (middle). Total CDH1 levels were detected by immunoblotting of the cell lysates with anti-HA (bottom). (**C**) Smad3 or Smad2 recruits the APC to SnoN in response to activation of TGFβ signaling. Flag-SnoN and HA-Smad were co-transfected into 293T cells in the absence or presence of a constitutive active ($T^{204}D$)TβRI (TβRI*). The APC was isolated from the nuclear extract by immunoprecipitation with anti-Cdc27 and anti-Cdc16 antisera, and the associated SnoN or Smad3 was detected by immunoblotting with anti-Flag or anti-HA as labeled (top two panels). Total APC levels were detected by immunoblotting with anti-Cdc27 and anti-Cdc16 (third and fourth panels from the top). Total SnoN and Smad3 levels were detected by immunoblotting of the cell lysates with anti-Flag and anti-HA (bottom two panels). (**D**) TGFβ induces formation of an endogenous Smad3/SnoN/APC complex. Nuclear extracts were prepared from Ba/F3 cells that were stimulated with TGFβ for 20 min. Smad3 and the APC associated with SnoN was isolated from these nuclear extracts by immunoprecipitation with anti-SnoN and detected by western blotting with anti-Smad3, anti-Cdc27 or anti-Cdc16 antibodies. Controls for the levels of total Smad3, SnoN and APC in the nuclear extracts are shown. (**E**) In vitro reconstituted ubiquitination reactions. In vitro ubiquitin reactions were carried out with the purified Flag-SnoN, Flag-Smad3 or Flag-Smad2, GST-ubiquitin and the indicated ubiquitin enzymes as described in the materials and methods. Ubiquitinated SnoN was detected by western blotting with anti-SnoN and anti-

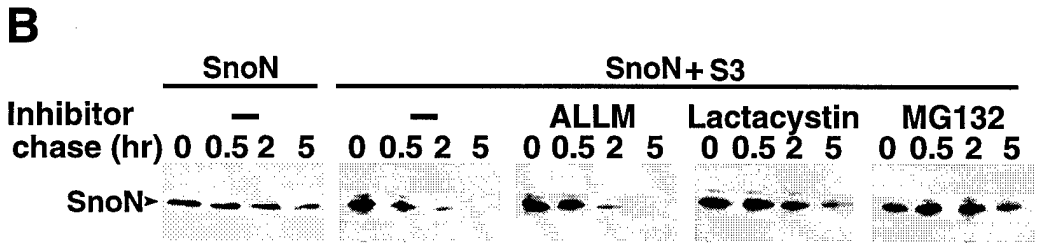
Flag antisera. (top panel). The membrane was re-blotted with anti-Flag (middle panel) to measure the level of SnoN and Smad3 in each reaction, and with anti-Cdc27 and anti-Cdc16 to determine the amounts of APC in each reaction (bottom two panels). * denotes a nonspecific band recognized by anti-SnoN antibody.

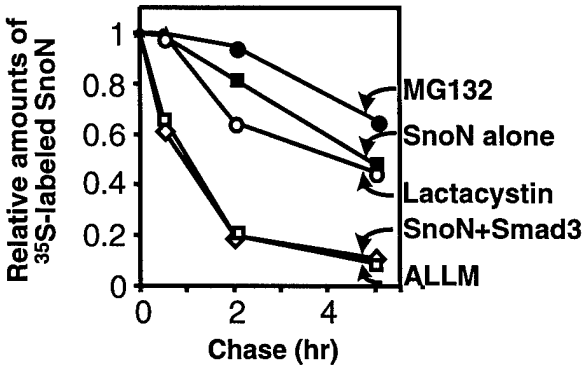
Figure 7. Stabilization of SnoN enhances its ability to block TGFβ signaling. (**A**) Ba/F3 cells stably expressing wildtype SnoN, SnoN 1-366 or SnoN K6-8R were stimulated with (lanes 3, 5 and 7) or without (lanes 1, 2, 4 and 6) TGFβ1 for 30 min. Flag-SnoN was isolated by immunoprecipitation with anti-Flag antiserum followed by immunoblotting with anti-Flag. (**B**) Growth inhibition assay. The same stable Ba/F3 cell lines shown in (A) were incubated for 4 days with various concentrations of TGFβ1 as indicated. The growth of cells was quantified by cell counting and compared with the growth of unstimulated cells.

Figure 8. Model of ubiquitination and degradation of SnoN.

Stroschein/Fig 1







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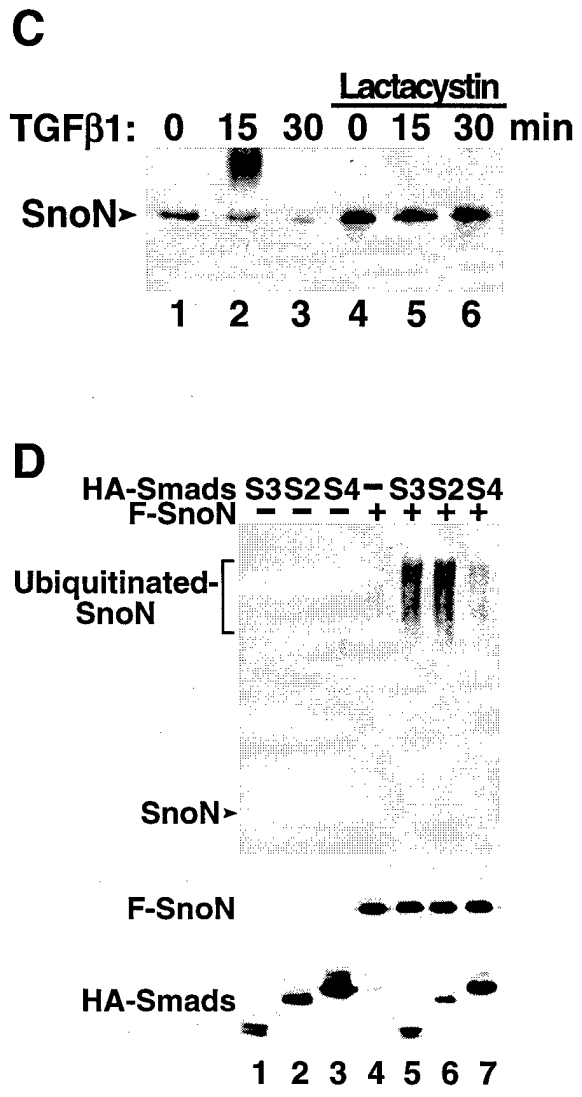

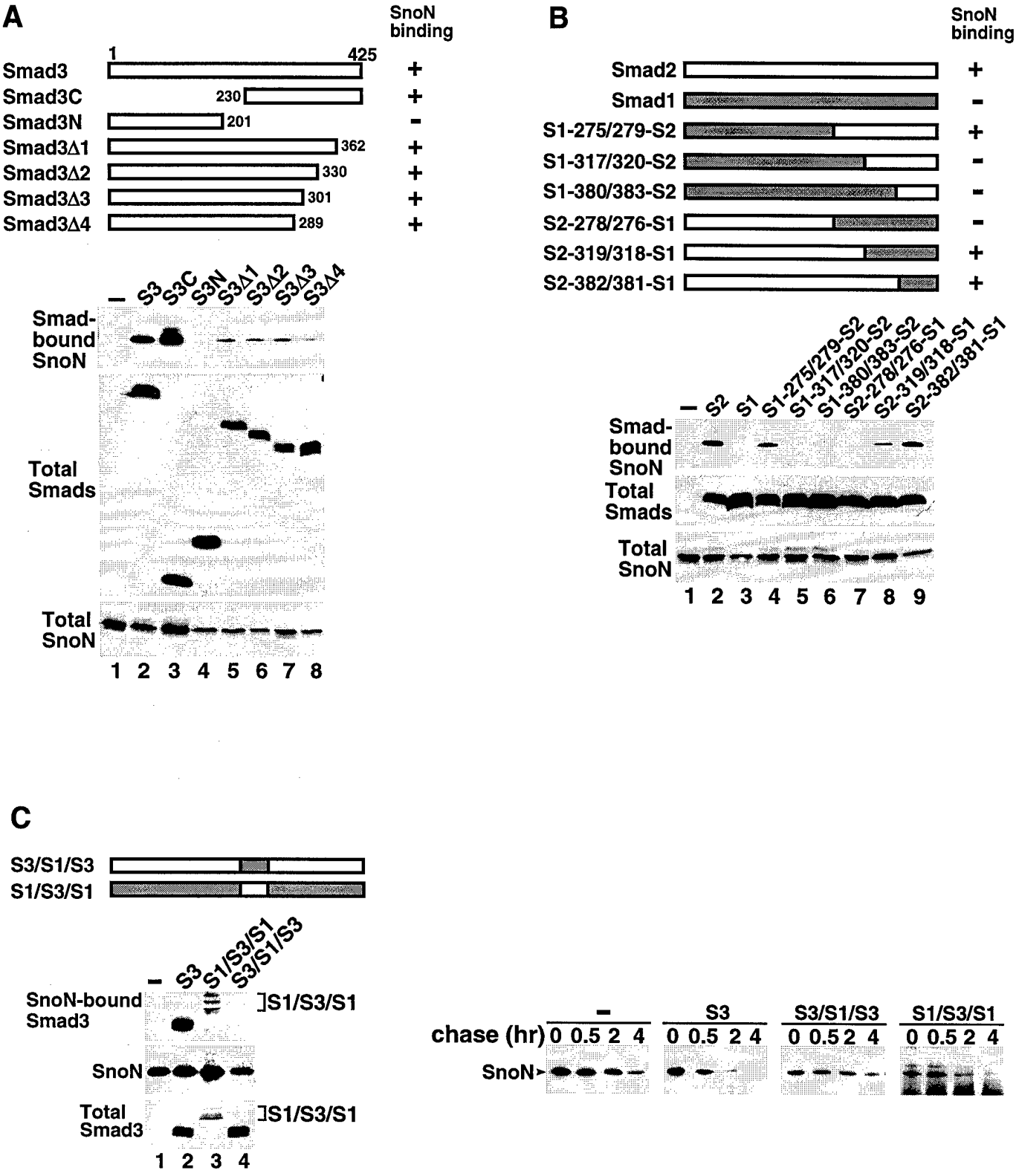
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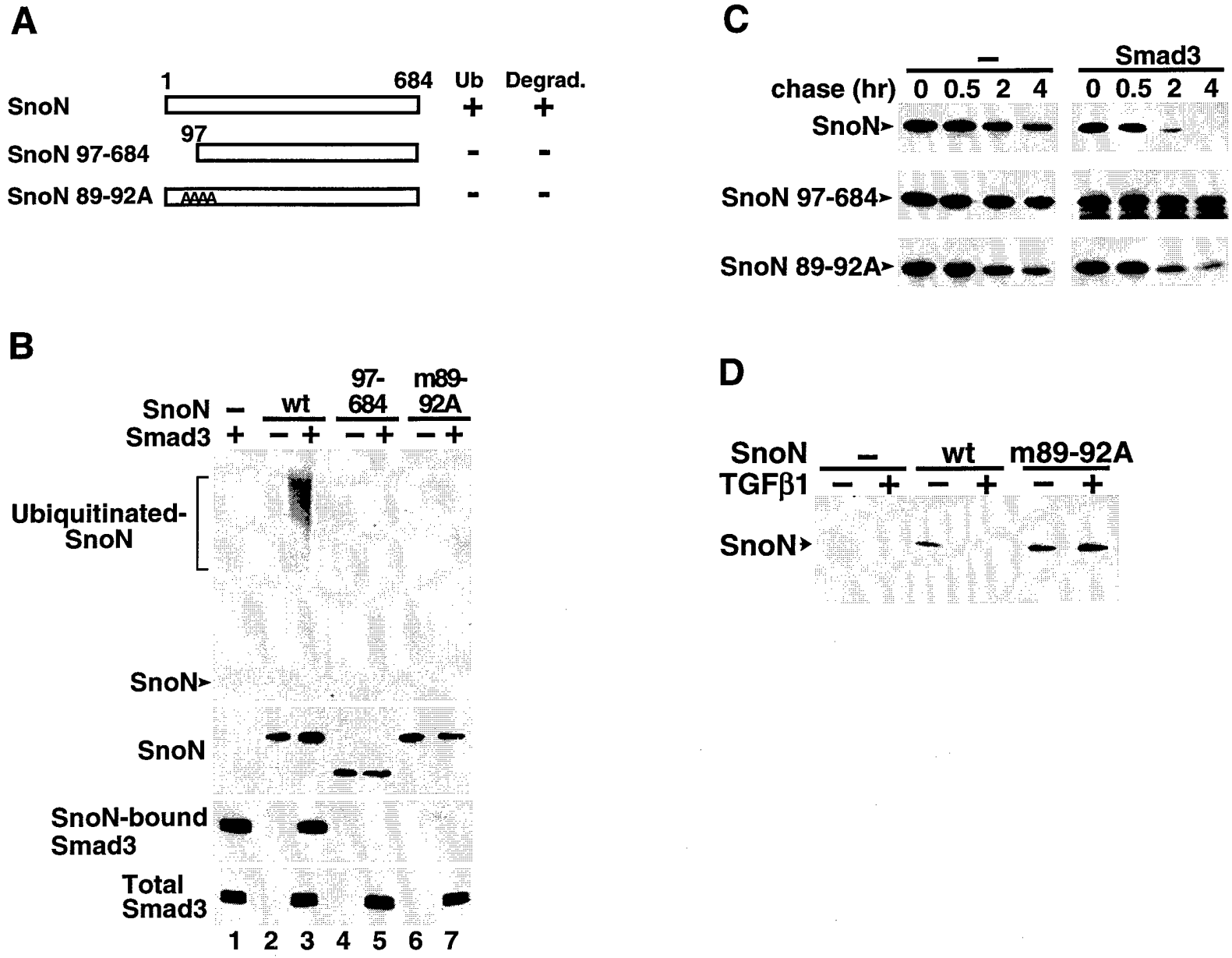
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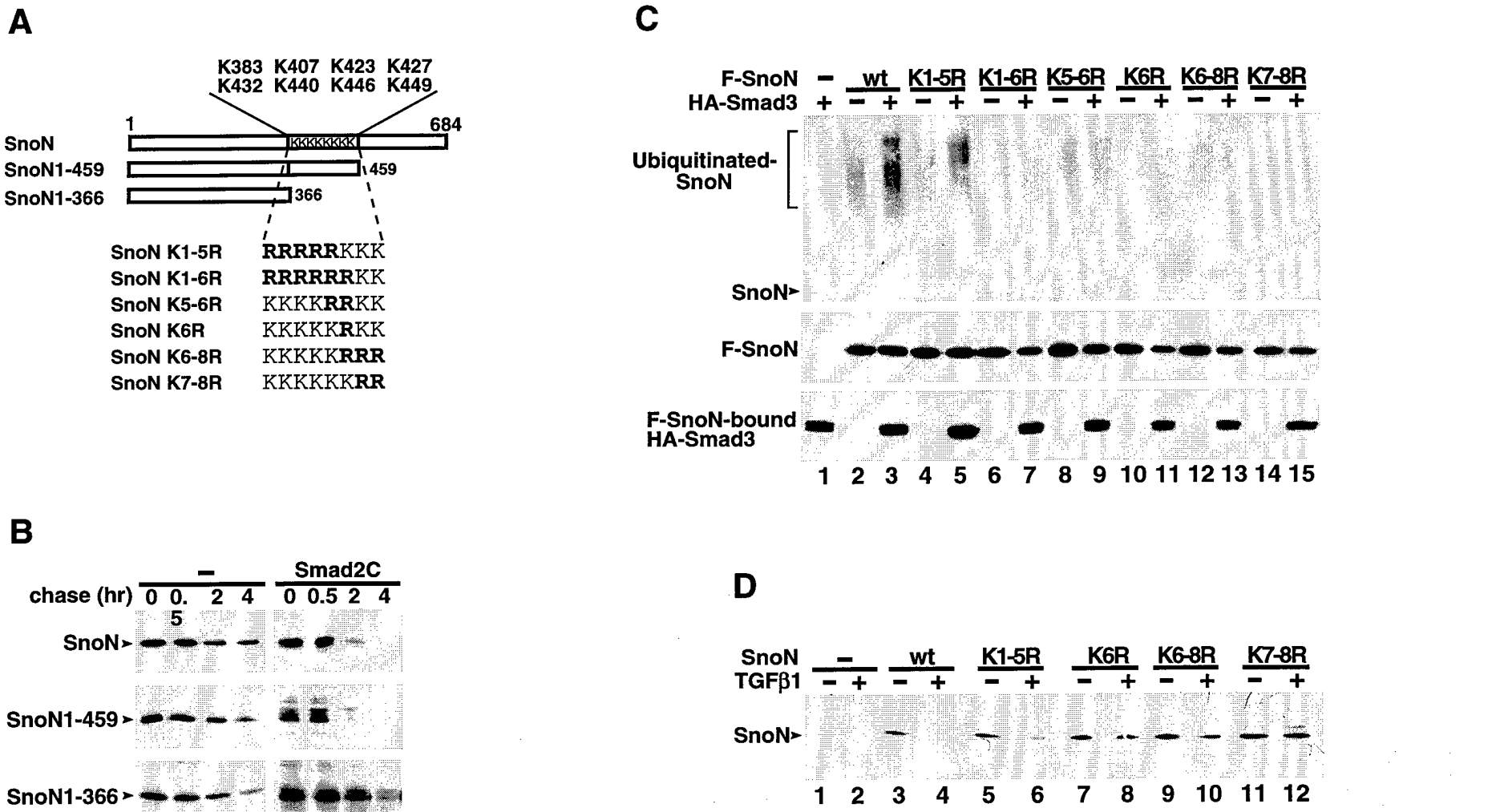
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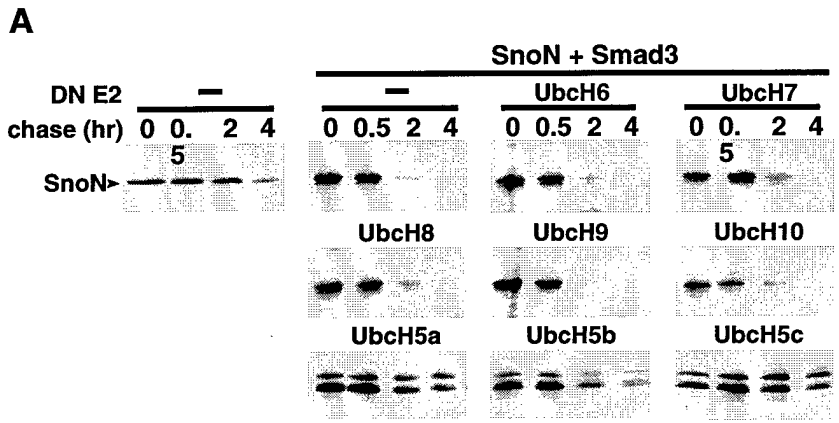
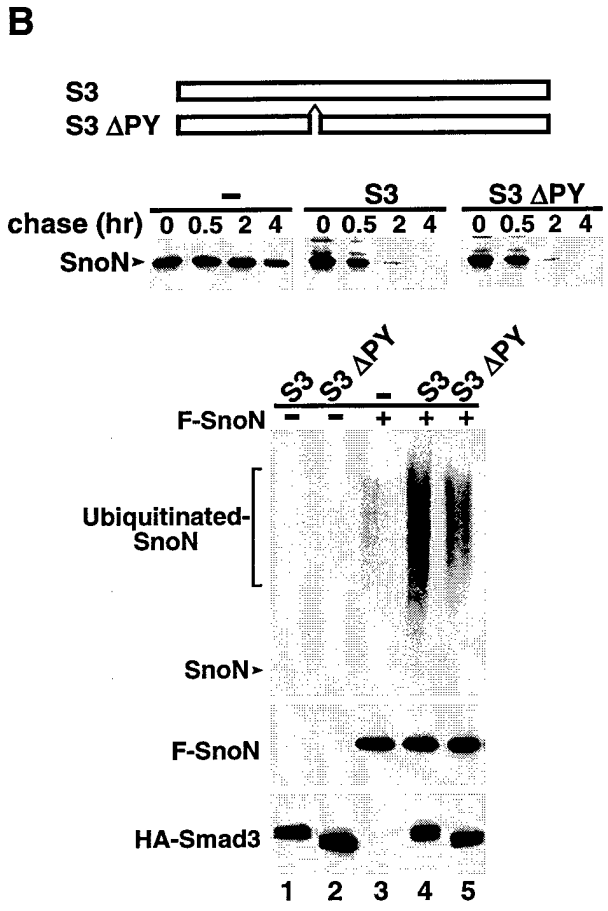
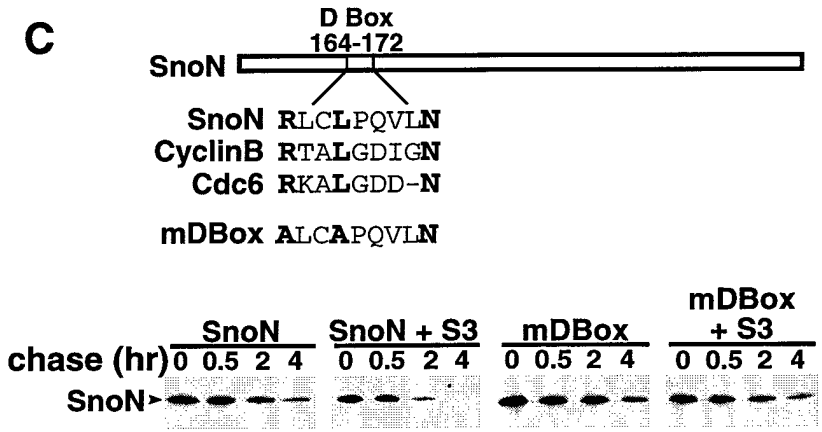
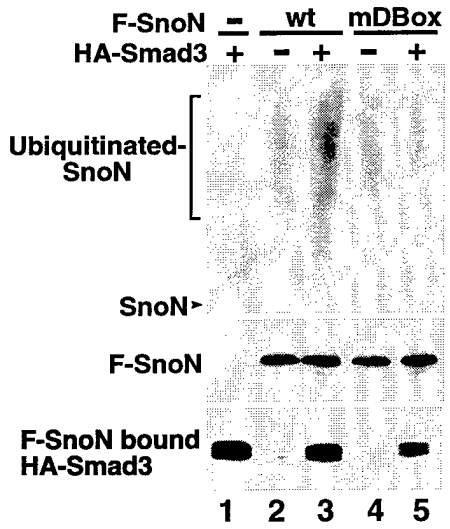
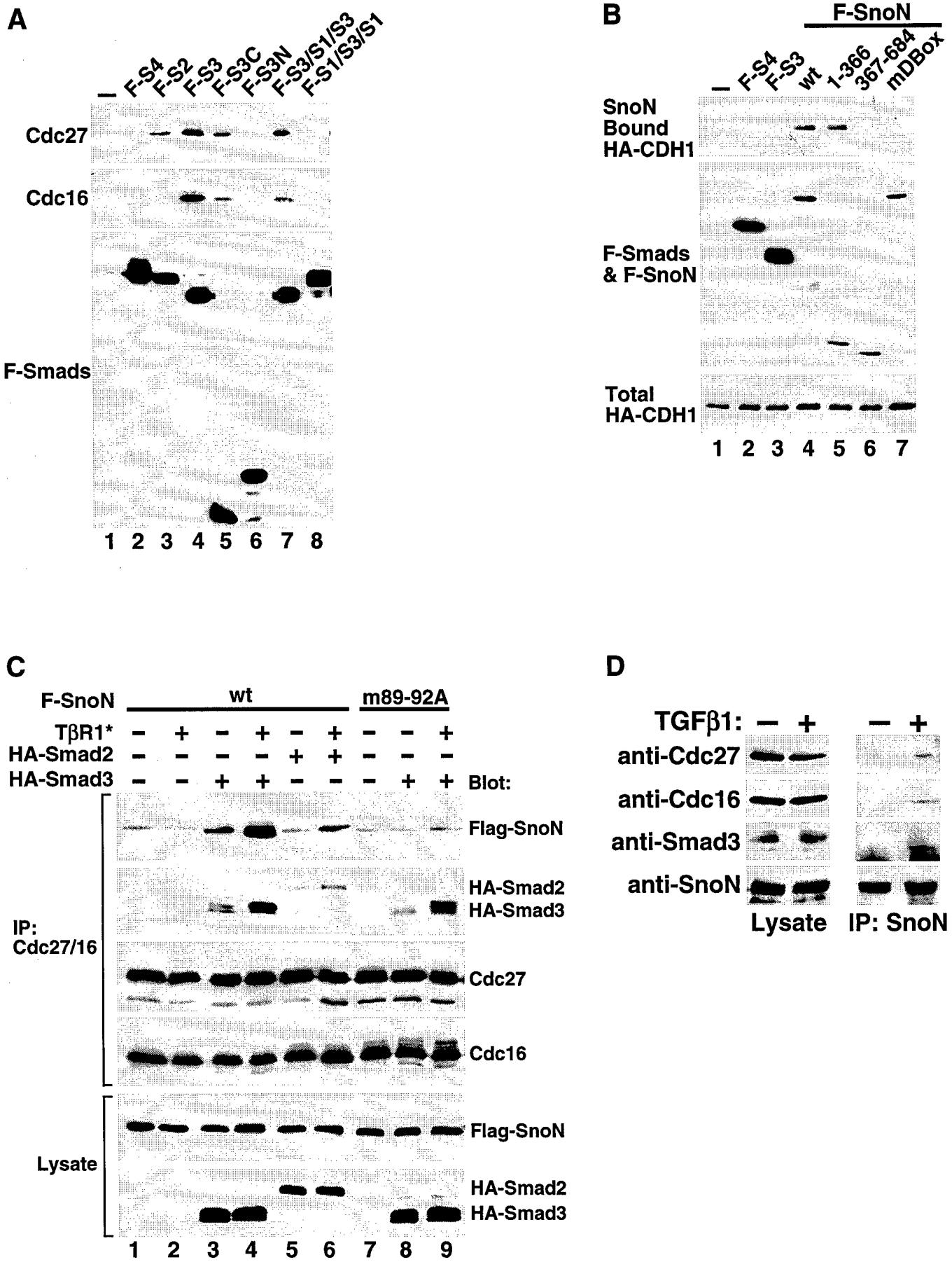
SnoN K6R KKKKKRKK
SnoN K6-8R KKKKKRRR
SnoN K7-8R KKKKKKRR
B
chase (hr) 0 0.5 2 4
Smad2C 0 0.5 2 4
SnoN
SnoN1-459
SnoN1-366
C
F-SnoN – wt K1-5R K1-6R K5-6R K6R K6-8R K7-8R
HA-Smad3 + – + – + – + – + – + – + – +
Ubiquitinated-SnoN
SnoN
F-SnoN
F-SnoN-bound HA-Smad3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
SnoN – wt K1-5R K6R K6-8R K7-8R
TGFβ1 – + – + – + – + – + – + – +
SnoN
1 2 3 4 5 6 7 8 9 10 11 12


A
SnoN + Smad3
DN E2
chase (hr)
0 0.5 2 4
SnoN
UbcH6
UbcH7
UbcH8
UbcH9
UbcH10
UbcH5a
UbcH5b
UbcH5c

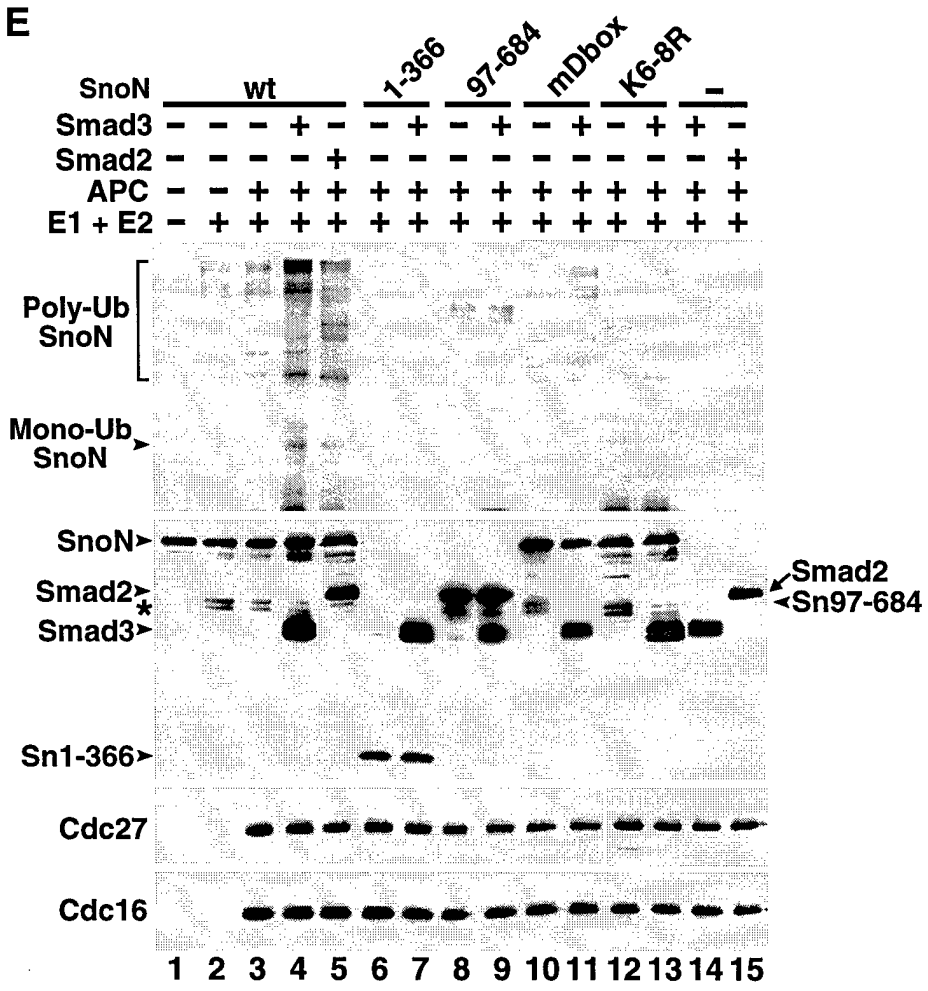

B
S3
S3 ΔPY
chase (hr)
SnoN
F-SnoN
HA-Smad3
Ubiquitinated-SnoN
1 2 3 4 5


C
D Box
164-172
SnoN
SnoN RLCLPQVLN
CyclinB RTALGDIGN
Cdc6 RKALGDD-N
mDBox ALCAPQVLN
chase (hr)
SnoN
SnoN + S3
mDBox
mDBox + S3
0 0.5 2 4
SnoN


F-SnoN
wt
mDBox
HA-Smad3
Ubiquitinated-SnoN
SnoN
F-SnoN
F-SnoN bound HA-Smad3
1 2 3 4 5

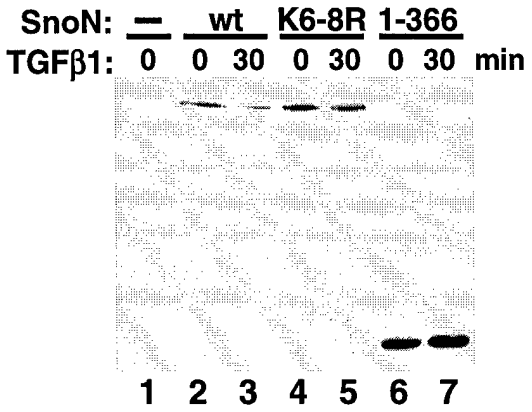

A
— F-S4 F-S2 F-S3 F-S3C F-S3N F-S3/S1/S3 F-S1/S3/S1
Cdc27
Cdc16
F-Smads
1 2 3 4 5 6 7 8
B
F-SnoN
— F-S4 F-S3 wt 1-366 367-684 mDBox
SnoN Bound HA-CDH1
F-Smads & F-SnoN
Total HA-CDH1
1 2 3 4 5 6 7
C
F-SnoN wt m89-92A
TβR1* – + – + – + – – +
HA-Smad2 – – – – + + – – –
HA-Smad3 – – + + – – – + +
Blot:
IP: Cdc27/16
Flag-SnoN
HA-Smad2
HA-Smad3
Cdc27
Cdc16
Lysate
Flag-SnoN
HA-Smad2
HA-Smad3
1 2 3 4 5 6 7 8 9
D
TGFβ1: – + – +
anti-Cdc27
anti-Cdc16
anti-Smad3
anti-SnoN
Lysate
IP: SnoN

Stroschein/Fig 6

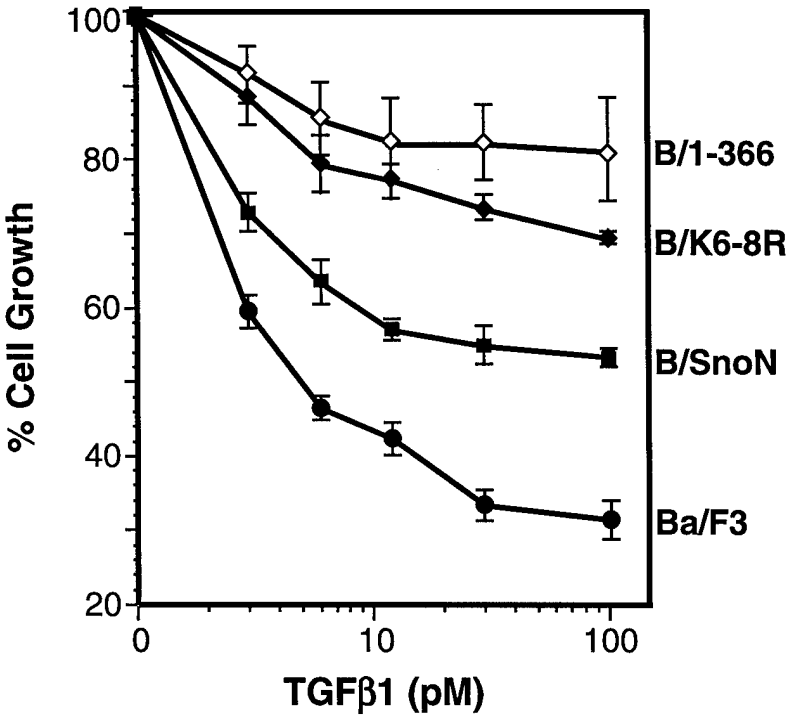


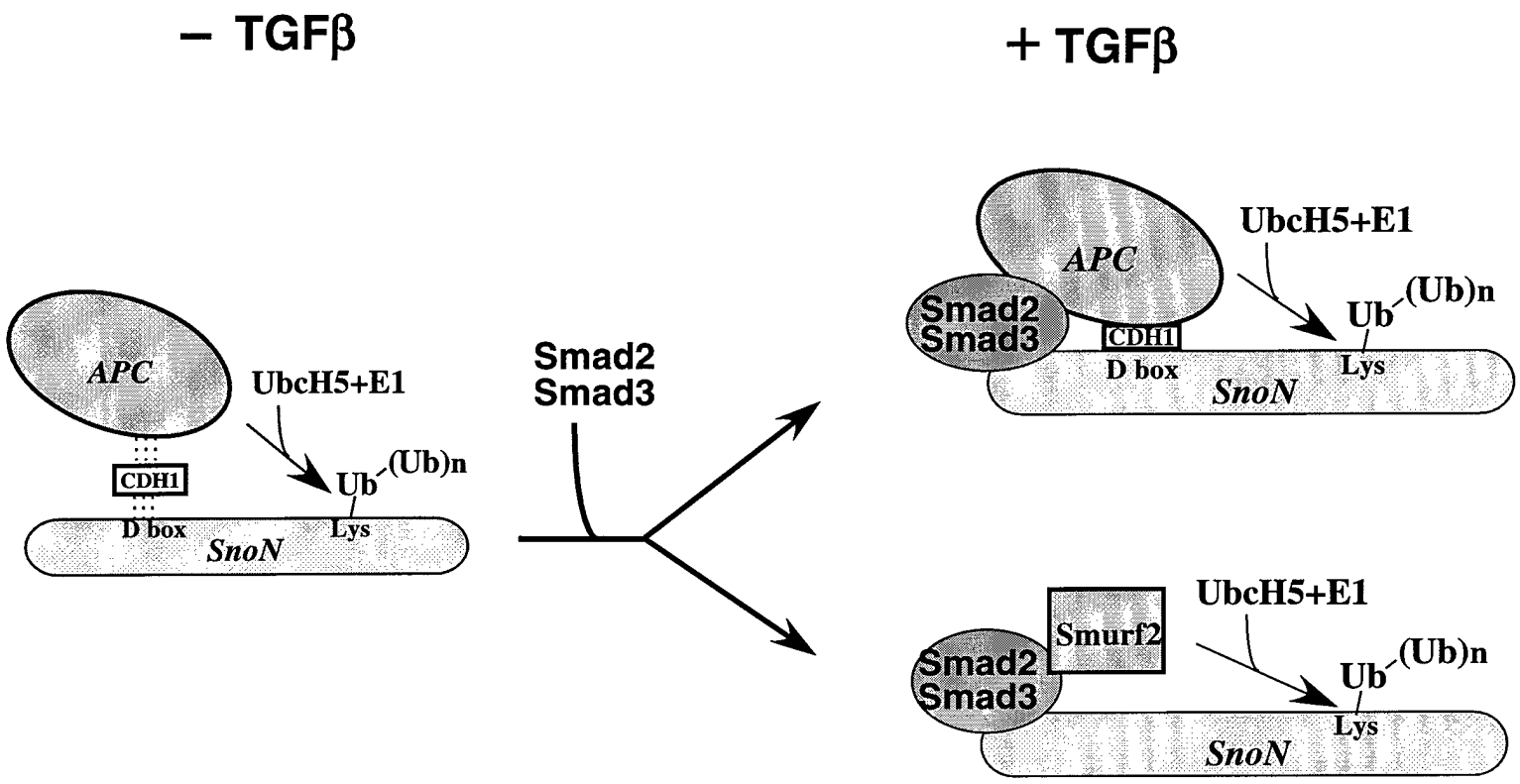
Stroschein/Fig 7

A



B




– TGFβ
+ TGFβ
APC
UbcH5+E1
CDH1
Ub
(Ub)n
D box
Lys
SnoN
Smad2
Smad3
Smurf2