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The Anaphase-Promoting Complex Mediates TGF-β Signaling by Targeting SnoN for Destruction

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Summary

Degradation of SnoN is thought to play an important role in the transactivation of TGF-β responsive genes. We demonstrate that the anaphase-promoting complex (APC) is a ubiquitin ligase required for the destruction of SnoN and that the APC pathway is regulated by TGF-β. The destruction box of SnoN is required for its degradation in response to TGF-β signaling. Furthermore, the APC activator CDH1 and Smad3 synergistically regulate SnoN degradation. Under these circumstances, CDH1 forms a quaternary complex with SnoN, Smad3, and APC. These results suggest that APC^{CDH1} and SnoN play central roles in regulating growth through the TGF-β signaling system.

Introduction

TGF-β signals mediated by Smad proteins are involved in regulating a variety of important biological effects including cell growth, differentiation, matrix production, and apoptosis (Massagué and Wotton, 2000). The transduction of extracellular signals to the nucleus is accomplished by the sequential association of type II and type I receptors and Smad protein cascades (Derynck et al., 1998; Massagué, 1998). Binding of ligands to the type II receptor causes phosphorylation of the cytoplasmic kinase domain of the type I receptor, leading to phosphorylation of Smad2 or Smad3 on the carboxy-terminal SSXS residues (Macias-Silva et al., 1996). Once phosphorylated, Smad2 or Smad3 forms oligomers with Smad4, translocates to the nucleus, and regulates the expression of downstream target genes such as the cyclin-dependent kinase inhibitors $p21^{WAF1/Cip1}$ and $p15^{INK4B}$ (Datto et al., 1995; Li et al., 1995; Pardali et al., 2000; Feng et al., 2000).

In the past two years, it has become clear that regulated proteolysis plays a critical role in regulating TGF-β signaling by targeting different components of this pathway for destruction by the 26 S proteasome. Once the signaling proteins are recruited for the transduction of a signal, they decline, presumably to reset the system to its initial state. Ubiquitin-dependent degradation targets components of the TGF-β pathway including cytoplasmic second messengers, transmembrane-bound receptors, and accumulated nuclear proteins. Smurfs, a group of C2-WW-HECT domain ubiquitin ligases, are involved in the clearance of the activated components through an interaction between their WW domains and PPXY motifs present on the substrate. Smad1 and Smad5, components of the BMP pathway, are degraded by Smurf1 (Zhu et al., 1999), whereas activated TGF-β receptors are removed by Smurf2 by associating with Smad7 after interferon γ stimulation (Kavsak et al., 2000). Degradation of nuclear accumulated Smad1 and Smad2 is also ubiquitin-mediated and targeted by Smurf2 (Zhang et al., 2001; Lin et al., 2000). While the above reactions can be considered part of an adaptation response returning the cell to its initial state, degradation steps have also been shown recently to be part of the initial signaling reaction. It is now thought that the turnover of specific inhibitors of transcription is required for induction of TGF-β-responsive genes. SnoN as well as Ski, two corepressors of transcription, are rapidly degraded upon stimulation of TGF-β1 in a ubiquitin-dependent manner (Sun et al., 1999; Stroschein et al., 1999). Recently, Smurf2 has been suggested to be involved in the degradation of SnoN in U4A/Jak1 cells (Bonni et al., 2001).

Several ubiquitin ligases, including the Smurfs, the SCF complexes, Ring finger proteins, and the APC (anaphase-promoting complex) are candidate E3s for SnoN ubiquitination. We show here that the APC, the E3 responsible for the metaphase/anaphase transition in mitosis, is an E3 ligase required for destruction of SnoN. SnoN contains a canonical destruction box (RXXL) at its NH_2 terminus, one of two sequence elements present in all APC substrates discovered to date (King et al., 1996; Pfleger and Kirschner, 2000). APC has been shown to be activated by either a single CDC20 gene or a family of CDH1 genes. These activators bind the substrate and have different but overlapping substrate specificities (Pfleger et al., 2001; Wan and Kirschner, 2001). The CDH1 protein becomes active at the end of mitosis, when it destroys the anaphase activator CDC20 and remains bound to the APC through G1 (Fang et al., 1998; Pfleger and Kirschner, 2000). The function of the G1 activity of APC^{CDH1} is unclear, as no convincing substrates have been identified. However, the presence of active APC in nondividing tissues has suggested a role for the APC outside of the cell cycle (Gieffers et al., 1999).

We have performed in vivo experiments in cultured cell lines and in vitro experiments in somatic cell extracts to demonstrate that SnoN contains a functional destruction box. In vitro reconstitution experiments in somatic cell extracts suggest that destruction of SnoN occurs through the APC pathway, that CDH1 and Smad3 synergistically regulate the turnover of SnoN, and that CDH1 forms a complex with SnoN in the presence of Smad3. Ectopic expression of dominant-negative CDH1 blocks APC-mediated SnoN degradation induced by TGF-β signaling. Our results suggest that APC-dependent degradation is activated in response to the TGF-β pathway.

Results

SnoN Fluctuates during the Cell Cycle, Suggesting Degradation through the APC Pathway

SnoN inhibits the transactivation of TGF-β-responsive genes (Sun et al., 1999; Stroschein et al., 1999). It turns

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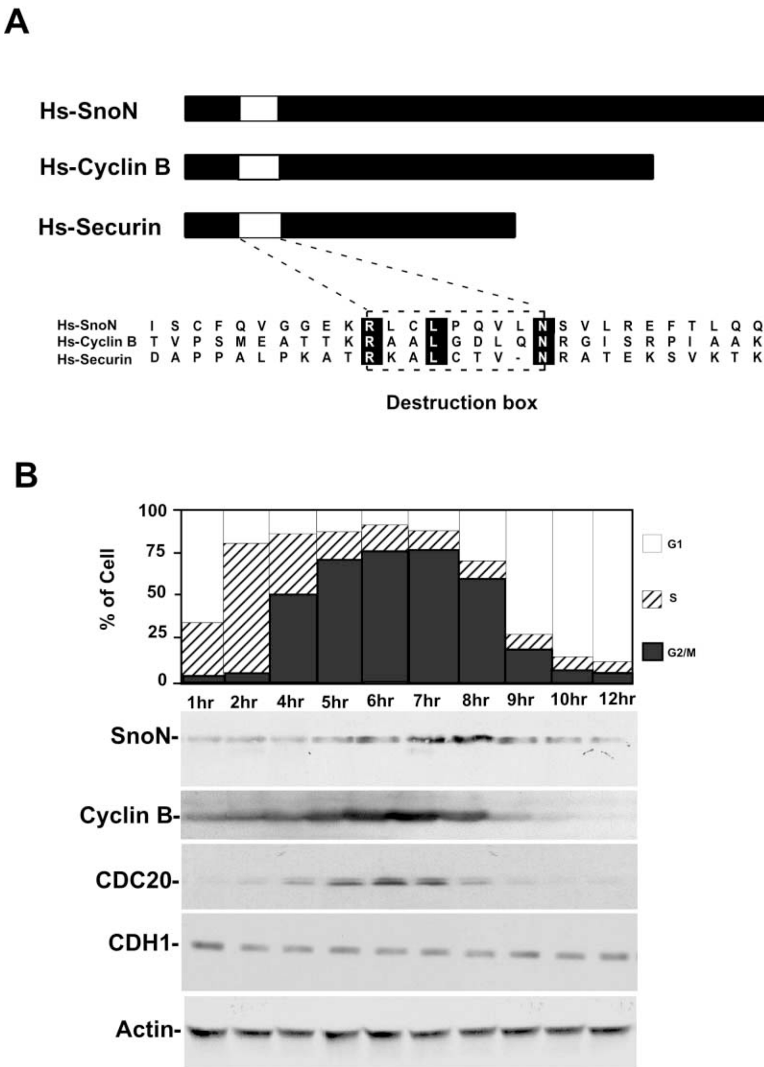

Figure 1. SnoN Contains a Destruction Box, and Its Protein Level Fluctuates during the Cell Cycle

(A) Alignment of SnoN with known APC substrates, Cyclin B and Securin. Similar to most APC substrates, SnoN contains a destruction box (RXXL) at its NH_2 terminus. The alignment was performed using the CLUSTAL W method. Hs, Human.

(B) SnoN levels fluctuate during the cell cycle. Hela S3 cells were synchronized at the G1/S boundary by a double thymidine block, and samples were taken at various time points. The top panel represents the percentage of cells in the G1, S, and G2/M phases of the cell cycle, as determined by FACS analysis. The lower panels show (from top to bottom) the protein levels of SnoN, Cyclin B, CDC20, CDH1, and Actin (as control) by immunoblotting.

over with a half-life of approximately 1 hr, and its turnover involves the ubiquitin pathway (Sun et al., 1999; Stroschein et al., 1999). To identify the possible ubiquitin protein ligase (E3) involved in SnoN degradation, we searched the SnoN sequence for known destruction motifs such as PEST, HECT, Ring finger, PY, or destruction box motifs (Jackson et al., 2000). SnoN does not contain PEST, HECT, Ring finger, or PY motifs. But like most APC substrates, it contains a destruction box (RXXL) at its NH_2 terminus (King et al., 1996) (Figure 1A). This result suggested that SnoN might be a substrate of the APC.

All known APC substrates play important roles in the cell cycle. The activity of APC is regulated by CDC20 and CDH1, where CDC20 associates with the APC in mitosis and CDH1 associates with the APC in late mitosis and in G1. To ask if SnoN might be a substrate for APC, we first measured the abundance of SnoN at various stages of the cell cycle. Hela S3 cells were synchronized at the G1/S boundary and released, extracts were prepared, and SnoN was detected by an anti-hSnoN monoclonal antibody. SnoN begins to accumulate at the onset of G2 and peaks at G2/M. Its level drops in late mitosis and remains low in G1 (Figure 1B). Unlike CDC20 and Cyclin B, the level of SnoN drops only about 3- to 4-fold during exit from mitosis. The abrupt drop in the levels of SnoN in mitosis and G1 suggests that the APC might be the E3 responsible for SnoN degradation. CDH1 would most likely be the activator of the APC for targeting SnoN, since CDH1 but not CDC20 is present during G1 when SnoN levels stay low (Figure 1B) (Fang et al., 1998).

Destruction of the SnoN Is Mediated by the APC Pathway

To test if SnoN is an APC substrate, we initially assayed the stability of SnoN in *Xenopus* egg extracts, as previously described (Pfleger and Kirschner, 2000). ^{35}S-labeled in vitro-translated SnoN was added to interphase or mitotic extracts. Aliquots were removed, and SnoN was detected by SDS-PAGE. As shown in Figure 2, SnoN is degraded rapidly in mitotic extracts with a half-life of approximately 50 min but is stable in interphase extracts. Most APC substrates are ubiquitinated in a D box-dependent manner. To ask whether the putative D box at the NH_2 terminus of SnoN is functional, we constructed two SnoN mutants: one that had

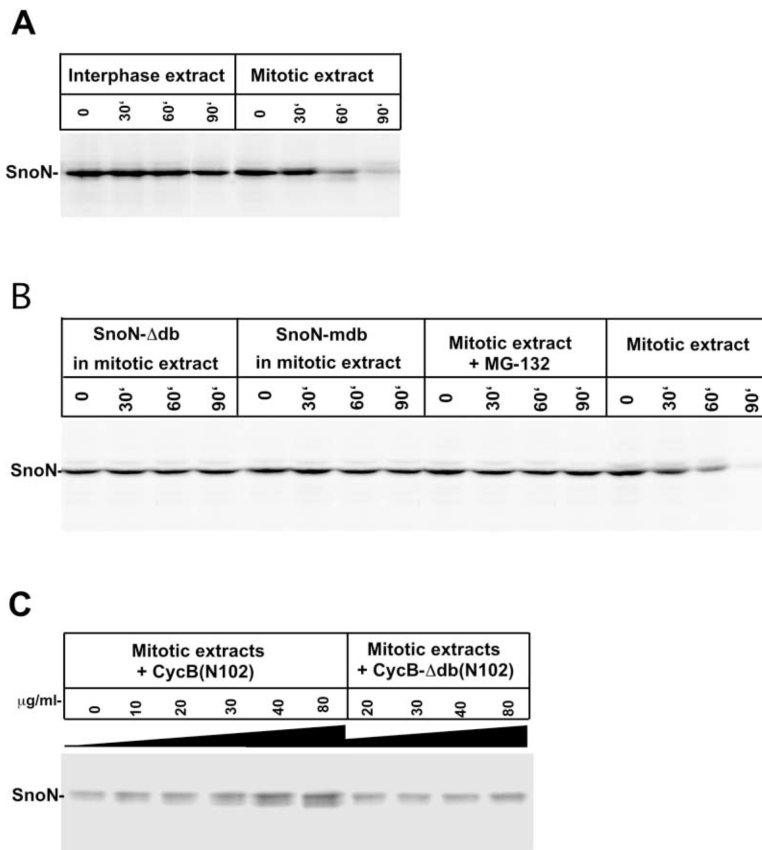

Figure 2. Degradation of SnoN Is Mediated by APC

(A) SnoN is rapidly degraded in mitotic *Xenopus* egg extracts but not in interphase extracts. ^{35}S-labeled in vitro-translated SnoN was added to *Xenopus* mitotic or interphase extracts. Aliquots were removed at the indicated times and resolved by SDS-PAGE followed by autoradiography.

(B) Deletion and point mutations of the destruction box stabilize SnoN in mitotic extracts; SnoN destruction requires the proteasome. Mutants of SnoN lacking destruction box (SnoNΔdb) or carrying alanine instead of the conserved arginine and lusine in D box (SnoN-mdb) were added to mitotic extracts. Degradation was measured as above. Degradation of wild-type SnoN was measured in the presence or in the absence of the proteasome inhibitor, MG-132 (150 μM).

(C) The degradation of SnoN is blocked by the addition of a destruction box-containing peptide. NH_2-terminal fragments (amino acids 1–102) of *Xenopus* Cyclin B or a control fragment lacking the D box (CycB-Δdb(N102)) were added to mitotic extracts and ^{35}S-labeled SnoN at the indicated concentration. After incubation for 90 min at room temperature, samples were analyzed by SDS-PAGE and autoradiography.

the D box deleted and one that had the conserved arginine and leucine residues at positions 164 and 167 replaced by alanine. As shown in Figure 2B, both mutant forms of SnoN were stable in mitotic extracts, confirming that the D box is required for degradation. Addition of MG-132 to a concentration of 150 μM also blocked the degradation (Figure 2B). Furthermore, SnoN was stabilized in the presence of an excess of an NH_2-terminal fragment of Cyclin B that contains a D box and therefore serves as a competitive inhibitor for APC-dependent degradation (Figure 2C) (McGarry and Kirschner, 1998). Taken together, these results suggest that proteolysis of SnoN occurs through the APC pathway and requires a functional destruction box sequence.

Regulation of the APC Activity by TGF-β Signaling

We have also developed a somatic cell extract system to study SnoN degradation. This system is able to detect the modification (degradation and phosphorylation) of the intervening components of the signal transduction pathway in response to ligand stimulation. Extracts were prepared from mink lung epithelial cells (Mv1Lu or ML cells) that had been exposed to TGF-β for various times; these cells are very responsive to TGF-β stimulation. ^{35}S-labeled in vitro-translated SnoN was added to extracts from these cells. As shown in Figure 3A, SnoN was degraded in extracts from cells previously stimulated by exposure to TGF-β. Under these conditions, SnoN had a half-life of approximately 60 min, which is similar to the half-life of SnoN in cultured mink lung cells in response to the TGF-β stimulation (Sun et al., 1999). Deletion or point mutation of the D box completely stabilized SnoN in TGF-β-stimulated extracts, as did addition of MG-132 (Figure 3B).

We can imagine two ways in which TGF-β signaling could cause an increased rate of SnoN degradation. In the first, TGF-β would, by well-known mechanisms, cause arrest of cell cycle progression in G1. The increase in the population of cells in G1 would be reflected in increased APC^{CDH1} activity, which is present in G1 and late in mitosis. In the second, TGF-β could regulate SnoN degradation in cells containing APC^{CDH1}. To distinguish between these two possibilities, we examined the ability to degrade SnoN in response to short exposure to TGF-β. Extracts prepared from cells exposed to TGF-β for various times were incubated with in vitro-translated and labeled SnoN. As shown in Figure 3C, degradation of SnoN was initiated 15 min after stimulation with TGF-β. The maximum activation was reached after 1 hr of TGF-β stimulation. We also analyzed the cell cycle profile by FACS analysis for Mv1Lu cells that were stimulated with TGF-β at time 0 or 15 min and found no change in the cell cycle profile. The FACS curve in both cases shows 50% in G1, 40% in S, and 10% in G2/M. Thus, the degradation of SnoN occurs without a measurable shift in the cell cycle distribution.

To ask whether the APC itself might be regulated by TGF-β signaling, we examined the degradation of other APC substrates in response to TGF-β. We tested the stability of Cyclin B and Securin in TGF-β-stimulated Mv1lu extracts. As shown in Figure 3D, Cyclin B and Securin were degraded very rapidly in TGF-β-stimulated

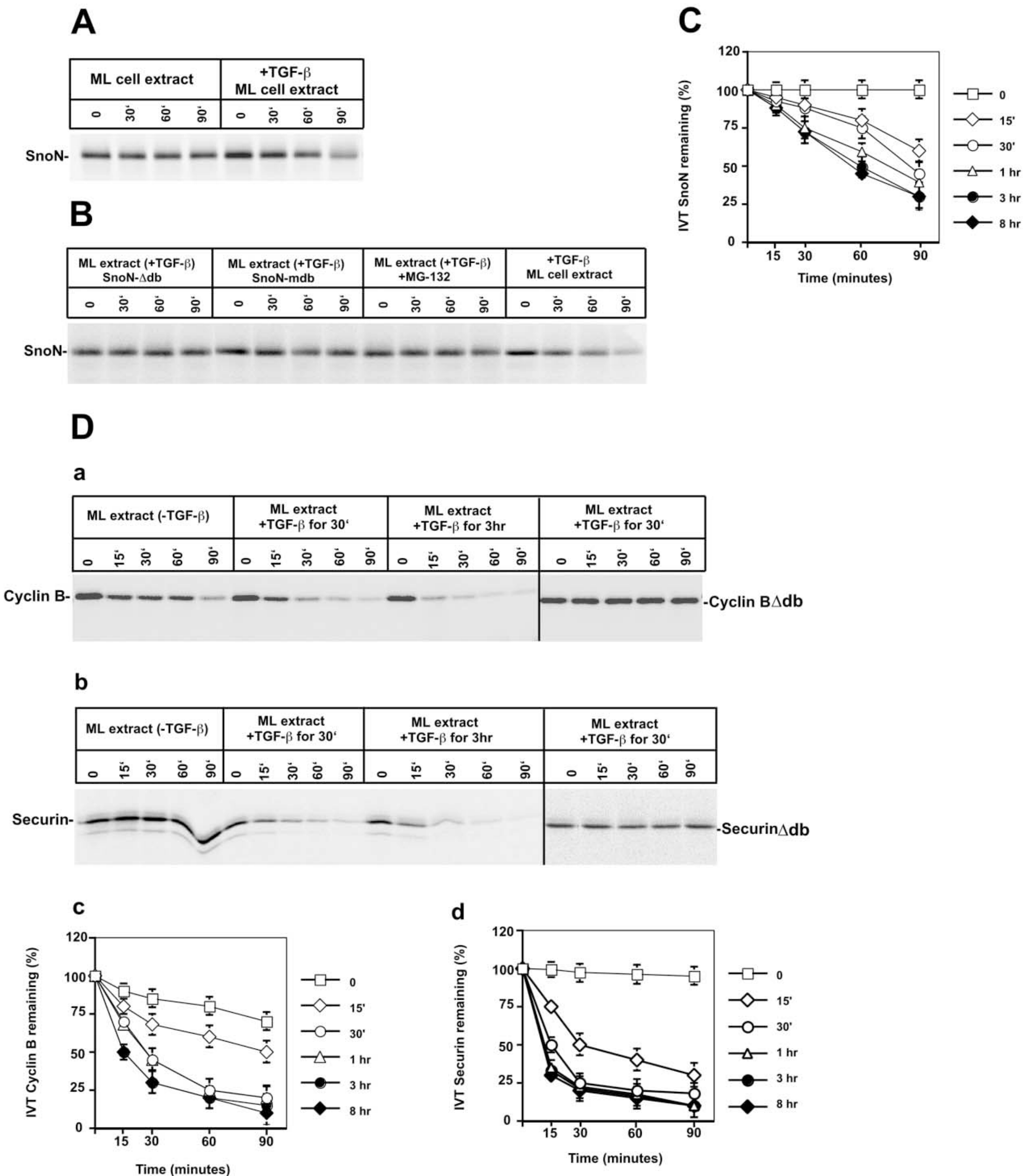

Figure 3. Degradation of SnoN and Other APC Substrates Is Regulated by TGF-β Signaling

(A) SnoN degradation requires TGF-β stimulation. Mink lung epithelial cells were either treated or not treated with 100 pM TGF-β for 30 min, and extracts were prepared. ^{35}S-labeled in vitro-translated SnoN was added to the extracts and supplemented with the degradation cocktail. Aliquots were removed at the indicated times and resolved by SDS-PAGE.

(B) The destruction box is required to mediate TGF-β-induced SnoN degradation. As in Figure 2B, the stabilization of SnoN was examined in the presence and absence of the proteasomal inhibitor (MG-132); a mutant of SnoN lacking the D box or carrying point mutation in the D box (SnoNmdb) was incubated in stimulated extracts or unstimulated.

(C) Susceptibility of SnoN to degradation is dependent on the time course of TGF-β stimulation. ^{35}S-labeled in vitro-translated SnoN was added to Mv1Lu extracts that had been stimulated with TGF-β for different lengths of time. Aliquots of cells were removed at the indicated times, and extracts were prepared.

(D) The rate of degradation of Cyclin B and Securin increases in response to TGF-β signaling. (a) Cyclin B is rapidly degraded in TGF-β-stimulated Mv1Lu extracts, whereas the D box-deleted Cyclin B is stable. (b) Securin is also rapidly degraded in extracts from TGF-β-stimulated cells, whereas the D box-deleted Securin is stable. (c) Cyclin B is degraded in extracts prepared from cells briefly exposed to TGF-β. (d) Securin degradation also increases in extracts prepared from cells briefly exposed to TGF-β.

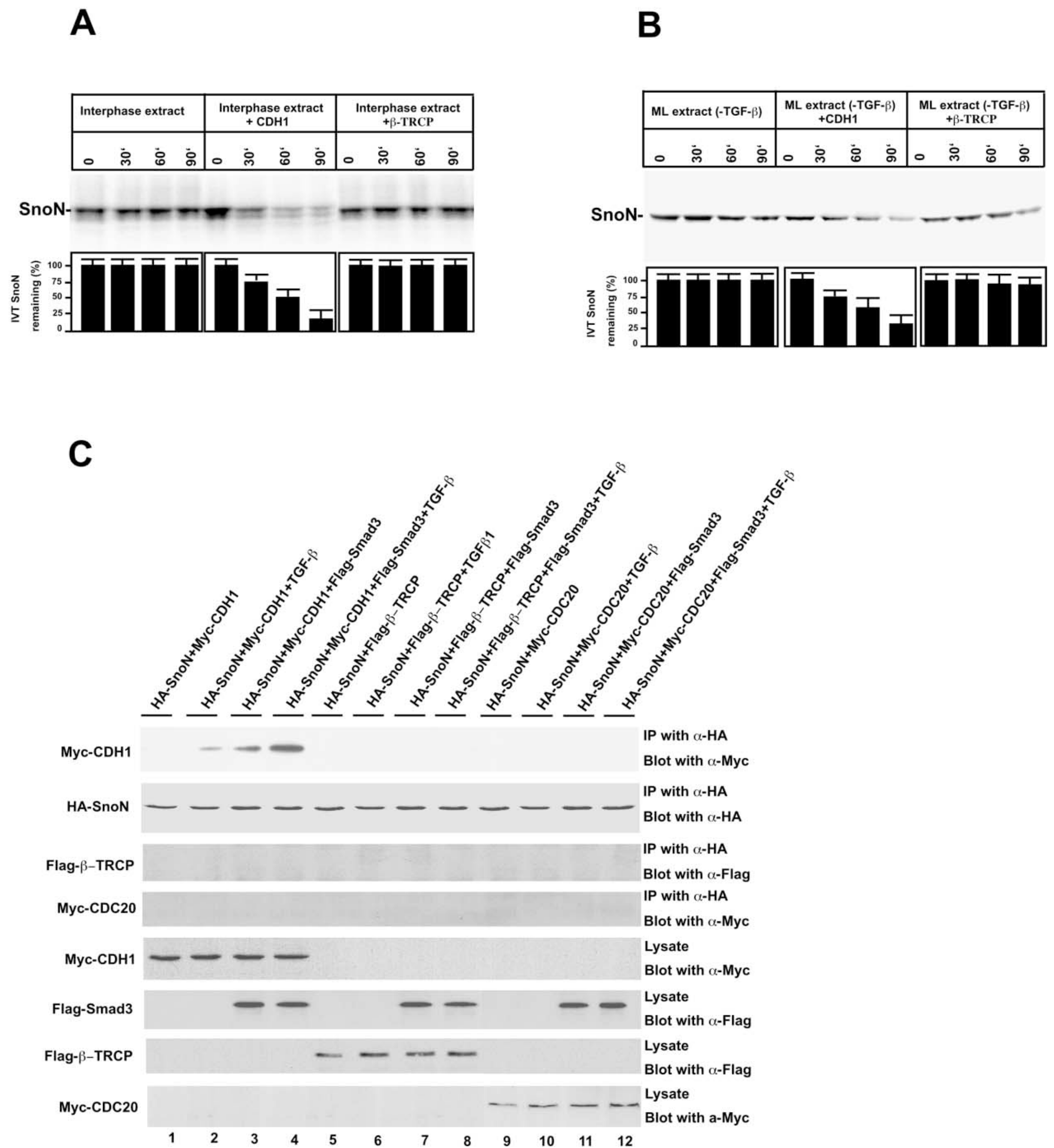

Figure 4. CDH1 Enhances the Destruction of SnoN and Forms a Complex with SnoN in the Presence of Smad3

(A) CDH1 triggers the degradation of SnoN in *Xenopus* interphase extracts. Approximately 100 ng (in 10 μl) of in vitro-translated CDH1 or the F box protein β-TRCP used as a control was added to *Xenopus* egg interphase extracts and preincubated for 30 min, at which point ^{35}S-labeled in vitro-translated SnoN was then added. Aliquots were removed at the indicated times and resolved by SDS-PAGE, and the levels of SnoN were measured.

(B) CDH1 enhances the degradation of SnoN in Mv1Lu extracts. Approximately 100 ng (10 μl) of in vitro-translated CDH1 or β-TRCP protein was added to 20 μl of Mv1Lu extracts made from unstimulated cells. ^{35}S-labeled IVT SnoN was added to extracts alone with the degradation cocktail. Aliquots were removed at the indicated times and resolved by SDS-PAGE, and the levels of SnoN were measured.

(C) CDH1 binds to SnoN in the presence of Smad3. Mv1Lu cells were cotransfected with the indicated combinations of the following expression vectors encoding: HA-SnoN/Myc-CDH1, HA-SnoN/Myc-CDH1/Flag-Smad3, HA-SnoN/Flag-β-TRCP, HA-SnoN/Flag-β-TRCP/Flag-Smad3, HA-SnoN/Myc-CDC20, or HA-SnoN/Myc-CDC20/Flag-Smad3. Transfected cells were preincubated with the proteasomal inhibitor MG-132 or vehicle (DMSO) for 1 hr before stimulation with TGF-β. HA-SnoN complexes were recovered using an anti-HA matrix. The interaction between SnoN and CDH1, β-TRCP, or CDC20 was judged by protein immunoblotting with anti-Myc or anti-Flag antibodies. Expression levels of HA-SnoN, Myc-CDH1, Flag-Smad3, Flag-β-TRCP, and Myc-CDC20 were determined by Western blotting.

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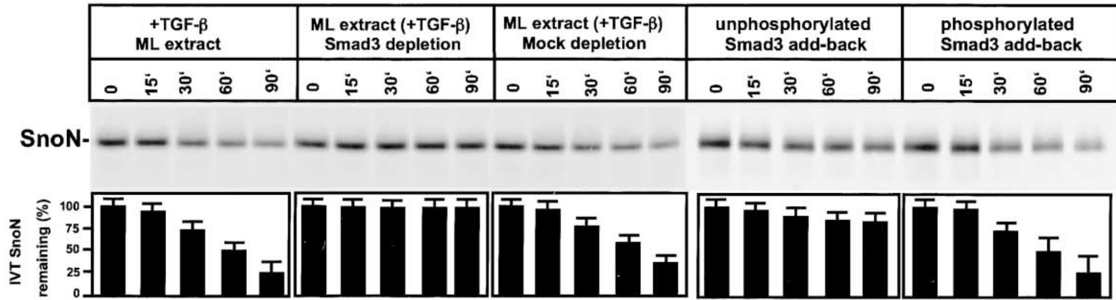

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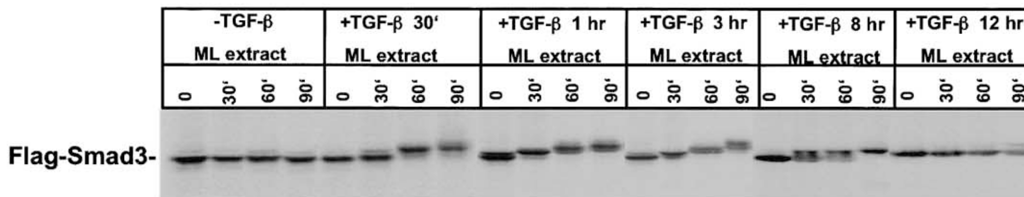

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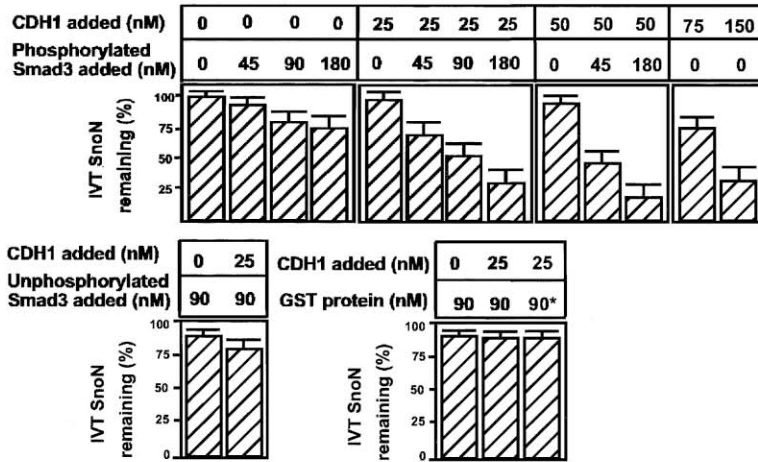

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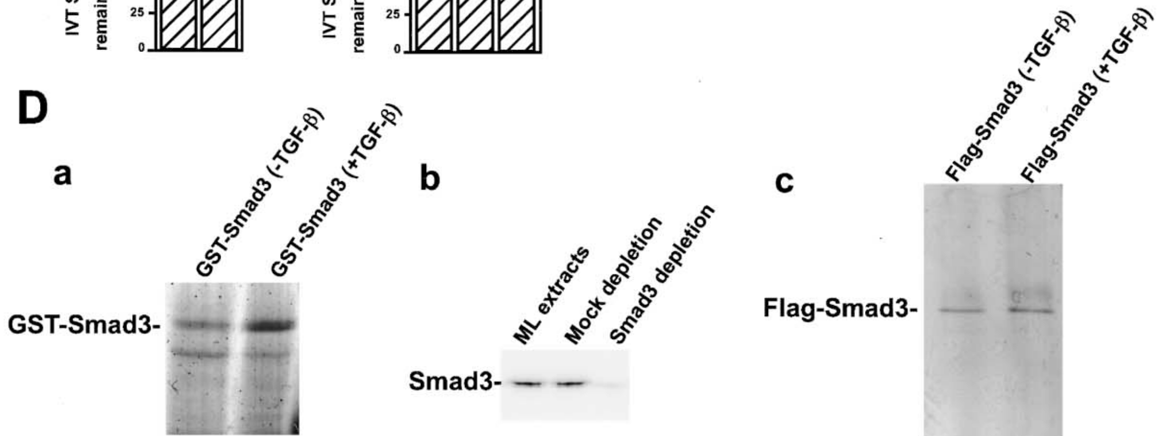

Figure 5. The Respective Contribution of Smad3 and CDH1 to SnoN Degradation

(A) Smad3 depletion prevents the destruction of SnoN. ^{35}S-labeled SnoN was added to extracts from TGF-β-stimulated Mv1Lu extracts. Anti-Smad3 or control beads were added to the extracts, incubated, and removed. Aliquots were removed at the indicated times and the proteins resolved by SDS-PAGE. Rescue experiments were performed by adding back purified, activated, or inactive Smad3 to the extracts after depletion.

Mv1Lu extracts but not in unstimulated extracts. Moreover, mutation of the respective destruction box sequences of Cyclin B and Securin stabilized them in TGF-β-stimulated Mv1Lu extracts (Figure 3D, a and b). Time course experiments showed that the APC-dependent degradation of Cyclin B and Securin was even more sensitive to TGF-β signaling than was SnoN (Figure 3D, c and d). In summary, these results demonstrate that SnoN is an APC substrate and that the APC pathway is activated after 15 min of TGF-β signaling before a detectable change in cell cycle profile.

CDH1 Enhances the Destruction of SnoN and Forms a Complex with SnoN in the Presence of Smad3

To test the effects of CDH1 on the degradation of SnoN, we added CDH1 or, as a control, the F box protein β-TRCP, to *Xenopus* interphase extracts or to Mv1Lu extracts prepared from cells exposed or not exposed to TGF-β. As shown in Figures 4A and 4B, addition of CDH1 triggered the degradation of SnoN in both interphase extracts and Mv1Lu extracts (in the absence of TGF-β), while β-TRCP had no effect.

Recent studies have shown that CDH1 binds directly to APC substrates (Pfleger et al., 2001). Smad3 has been demonstrated as a major signaling transducer in the TGF-β pathway. It has been shown that Smad3 interacts with SnoN when the TGF-β receptor is active (Sun et al., 1999). We wanted to know whether CDH1 interacts with SnoN and whether this interaction was dependent on the presence of Smad3 and/or stimulation with TGF-β in Mv1Lu cells. We cotransfected Mv1Lu cells with combinations of expression vectors encoding differentially tagged versions of SnoN, CDH1, and Smad3. Similarly, we asked whether CDC20 binds to SnoN. β-TRCP served as a control. The transfected cells were preincubated with the proteasomal inhibitor MG-132 (50 μM) or vehicle (DMSO) for 1 hr before stimulation with TGF-β (or mock treatment). Thirty minutes thereafter, the interaction between SnoN and CDH1, β-TRCP, or CDC20 was examined by immunoprecipitation and Western blotting (Figure 4C). In the absence of TGF-β stimulation, the association of SnoN with CDH1 was barely detectable. Cotransfection of Smad3 enhanced the interaction of CDH1 and SnoN. However, the optimal condition for coimmunoprecipitation of CDH1 and SnoN was with TGF-β-stimulated cells that expressed Smad3. In contrast, neither β-TRCP nor CDC20 associated with SnoN under any of these conditions. These results suggested that CDH1 is a substrate-specific activator of APC-mediated SnoN degradation and that CDH1 binds to SnoN in the presence of Smad3; this interaction is enhanced upon stimulation with TGF-β. These findings are consistent with recent results from Luo and colleagues demonstrating that Smad3 is required to target SnoN for degradation (Stroschein et al., 2001).

Smad3 Is Required for Mediating SnoN Degradation by CDH1

Because overexpression experiments can generate nonphysiological interactions, we wished to confirm the role of Smad3 in APC-mediated SnoN degradation by depleting endogenous Smad3 from Mv1Lu extracts. In mock-depleted extracts, SnoN was rapidly degraded. In contrast, degradation of SnoN was blocked in Smad3-depleted extracts (Figure 5A). We also demonstrated that Smad3 is activated by incubation in extracts from TGF-β-stimulated Mv1Lu cells. The phosphorylation of Smad3 can be detected after 15 min of stimulation and continues for 8 hr in Mv1Lu extracts (+TGF-β) (Figure 5B). We purified activated in vitro-translated Flag-Smad3 from Mv1Lu extracts (+TGF-β) (Figure 5D, c) and added this back to the Smad3-depleted extracts. A silver stained gel of the Smad3 shows phosphorylated and unphosphorylated species of Smad3 but no other major bands (Figure 5D, c). As shown in Figure 5A, addition of activated Smad3 to the depleted extracts rescued the degradation of SnoN, while adding back the unphosphorylated Smad3 failed to restore significantly the degradation of SnoN. These experiments suggest that Smad3 is required for SnoN degradation by CDH1, though they do not rule out substoichiometric amounts of additional components that may associate with activated Smad3.

CDH1 and Smad3 Synergistically Regulate the Degradation of SnoN

There is a potential inconsistency in the data presented above. We have shown in *Xenopus* and in Mv1Lu extracts that addition of CDH1 alone can induce the degradation of SnoN. Furthermore, there is a cell cycle periodicity of SnoN levels in cells not exposed to TGF-β. Yet, in the rapid degradation of SnoN in Mv1Lu cells, there is a strong requirement for phosphorylated Smad3. The most likely explanation for these results is that Smad3 acts synergistically to promote CDH1-dependent degradation and that the requirement for Smad3 depends on absolute levels of CDH1 and perhaps other components. To address this synergism, we have titrated CDH1 and Smad3 independently and in combination and assayed the effect on the degradation of SnoN (Figure 5C). These

(B) In vitro-translated Smad3 is phosphorylated in TGF-β-stimulated Mv1Lu extracts. ^{35}S-labeled in vitro-translated SnoN was added to the TGF-β-stimulated Mv1Lu extracts. Phosphorylation of Smad3 was indicated by the retardation of mobility in the gel.

(C) The degradation of SnoN is synergistically regulated by CDH1 and Smad3. Calibrated samples of purified CDH1 and phosphorylated Smad3 at a known concentration were incubated with TGF-β-stimulated and unstimulated Mv1Lu extracts both containing in vitro-translated, labeled SnoN. Samples were collected at 90 min after incubation and resolved by SDS-PAGE. Loss of SnoN was measured by electrophoresis and autoradiography. * GST protein served as a negative control.

(D) (a) Coomassie-stained purified GST-Smad3 proteins after incubation with Mv1Lu extracts (−TGF-β or +TGF-β). (b) Immunodepletion of Smad3 from Mv1Lu extracts. Equal amounts of undepleted, mock-depleted, and Smad3-depleted Mv1Lu extracts were resolved by SDS-PAGE and immunoblotting with anti-Smad3 antibodies to demonstrate the efficiency of the depletion. Depletion was estimated to remove approximately 80% of the Smad3 protein. (c) Silver stain of purified Flag-tagged IVT-Smad3 protein after incubation with Mv1Lu extracts (−TGF-β or +TGF-β). Approximately 200 ng unlabeled IVT Smad3 was added to 50 μl TGF-β-stimulated Mv1Lu extracts and incubated at room temperature for 60 min. Smad3 was subsequently purified on an anti-Flag matrix and extensively washed.

experiments were performed in unactivated Mv1Lu extracts, where the rate of degradation is slow unless other components are added. The low levels of endogenous CDH1 in these extracts have little effect. In the absence of exogenously added CDH1, even high levels of phosphorylated Smad3 (Figure 5D, a) induced only a very limited degradation of SnoN (Figure 5C). Addition of only 150 nM CDH1 increased the degradation rate significantly. Combining CDH1 and Smad3 had a large synergistic effect. For example, extracts with 45 nM phosphorylated Smad3 on its own degraded less than 10% of SnoN in 90 min. Similarly, 50 nM CDH1 on its own promoted less than 10% degradation. However, both together resulted in degradation of 50% of SnoN. Yet high levels of CDH1 (150 nM) gave 70% degradation in the absence of phosphorylated Smad3. Unphosphorylated Smad3 at 90 nM had no measurable synergistic effect on CDH1, even while CDH1 was present at 90 nM (data not shown). These results demonstrate that phosphorylated Smad3 and CDH1 synergistically regulate the targeting of SnoN for degradation. There is no absolute requirement for Smad3, but at limited concentrations of CDH1 (<75 nM), phosphorylated Smad3 is required.

The APC Is Required for the Degradation of SnoN in Response to TGF-β Signals In Vivo

Pfleger et al. (2001) have recently shown that an amino-terminal 125 amino acid peptide of CDH1 is a dominant-negative reagent for APC-mediated protein degradation. To establish further that APC is the physiological ubiquitin protein ligase for SnoN, we examined the effect of this dominant-negative CDH1 on the degradation of SnoN in the presence of a TGF-β signal. We cotransfected the dominant-negative CDH1, HA-SnoN, or control constructs along with a GFP marker in Mv1Ln cells and isolated transfected cells by cell sorting. As shown in Figure 6A, overexpression of the dominant-negative mutant blocked the degradation of both exogenous and endogenous SnoN in response to TGF-β stimulation. Overexpression of control constructs which included the carboxy-terminal fragment of CDH1, CDH1, and β-TRCP had no effect on the SnoN level. These results demonstrate that APC is a physiological E3 for the degradation of SnoN in response to TGF-β signaling.

Expression of D Box-Deleted SnoN Inhibits TGF-β-Stimulated SnoN Degradation

To confirm that the APC degrades SnoN in vivo via the D box, we developed two derivatives of the transformed 293 kidney cell lines that carry ecdysone-inducible HA-tagged SnoN and HA-tagged nondegradable SnoN (No et al., 1996; Shi et al., 2001). After induction, the expression levels of HA-SnoN and HA-SnoNΔdb were only 2- to 3-fold higher than that of the endogenous SnoN (No et al., 1996; Shi et al., 2001) (data not shown). These lines do not respond to TGF-β due to low expression of the receptor, though they contain all of the important downstream components (Bonni et al., 2001). In order to study the degradation of SnoN induced by TGF-β in these stable lines, we transfected these lines with a constitutively active TGF-β type I receptor (TβR1*) (Sun et al., 1999). Cotransfection with GFP and sorting allowed us to select for cells containing the altered TGF-β receptor. The level of SnoN (or the Δdb mutant) was assessed 12 hr after induction with ecdysone. As shown in Figure 6B, the SnoN level drops considerably when TβR1* is expressed (compare lanes 1 and 2), whereas the expression of a control receptor (ALK3*-constitutively active BMP receptor) had no effect (compare lanes 5 and 6). In contrast to wild-type SnoN, the D box mutant is not degraded in the presence of TβR1* (compare lanes 3 and 4).

Furthermore, we determined the stability of HA-SnoN and HA-SnoNΔdb at various stages of the cell cycle. We also performed FACS analysis to monitor the cell cycle profile in response to expression of wild-type or nondegradable SnoN in these cell lines. Cells were synchronized at mitosis by a thymidine and nocodozole block with or without the inducing hormone (ponasterone-A), and extracts were prepared after release from nocodozole. Endogenous SnoN, HA-SnoN, and APC2 were detected by the appropriate antibodies (see Experimental Procedures). We found that the levels of both endogenous SnoN and HA-SnoN dropped about 4-fold at the onset of G1 and remained low in G1 phase (Figure 6D) while D box-mutated HA-SnoN was stable (Figure 6E). We also noticed that initiation of S phase occurred earlier in the cells expressing nondegradable SnoN (Figure 6E) but not in the cells expressing wild-type SnoN (Figures 6E and 6C). We conclude that the TGF-β pathway mediates the degradation of SnoN in an APC-dependent manner in vivo. In the absence of TGF-β, APC may also be involved in the oscillation of SnoN abundance during the cell cycle, and stabilization of SnoN may affect the G1/S transition.

Discussion

The TGF-β pathway has attracted considerable interest for its broad role in mediating both developmental events and cell cycle events (Massagué, 1998). In recent years, proteolysis has also emerged as an important component of TGF-β signaling. Two negative regulators in the pathway that inhibit TGF-β signaling, SnoN and Ski, have been shown to be regulated by proteolysis as well as by phosphorylation. These inhibitors of signaling are likely to be involved in both the signaling as well as the attenuation processes. While Smurf2 has also been shown to form a complex containing SnoN and potentially target it for degradation (Bonni et al., 2001), we noticed that SnoN contained a destruction box sequence similar to that found in all mitotic cyclins, suggesting that the anaphase-promoting complex, APC, was responsible for degradation. This was further supported by the mitotic-specific degradation of SnoN in *Xenopus* cell-free extracts, as well as the strong inhibitory effect on degradation of deleting the D box or the use of a D box-containing peptide as a competitor.

There are several potentially important implications of the involvement of APC, beyond identification of a ubiquitin protein ligase responsible for SnoN degradation. First, since APC is intimately tied to cell cycle control, a potential connection between TGF-β signaling and the cell cycle is suggested. Second, as a master regulator controlling several processes in the cell cycle

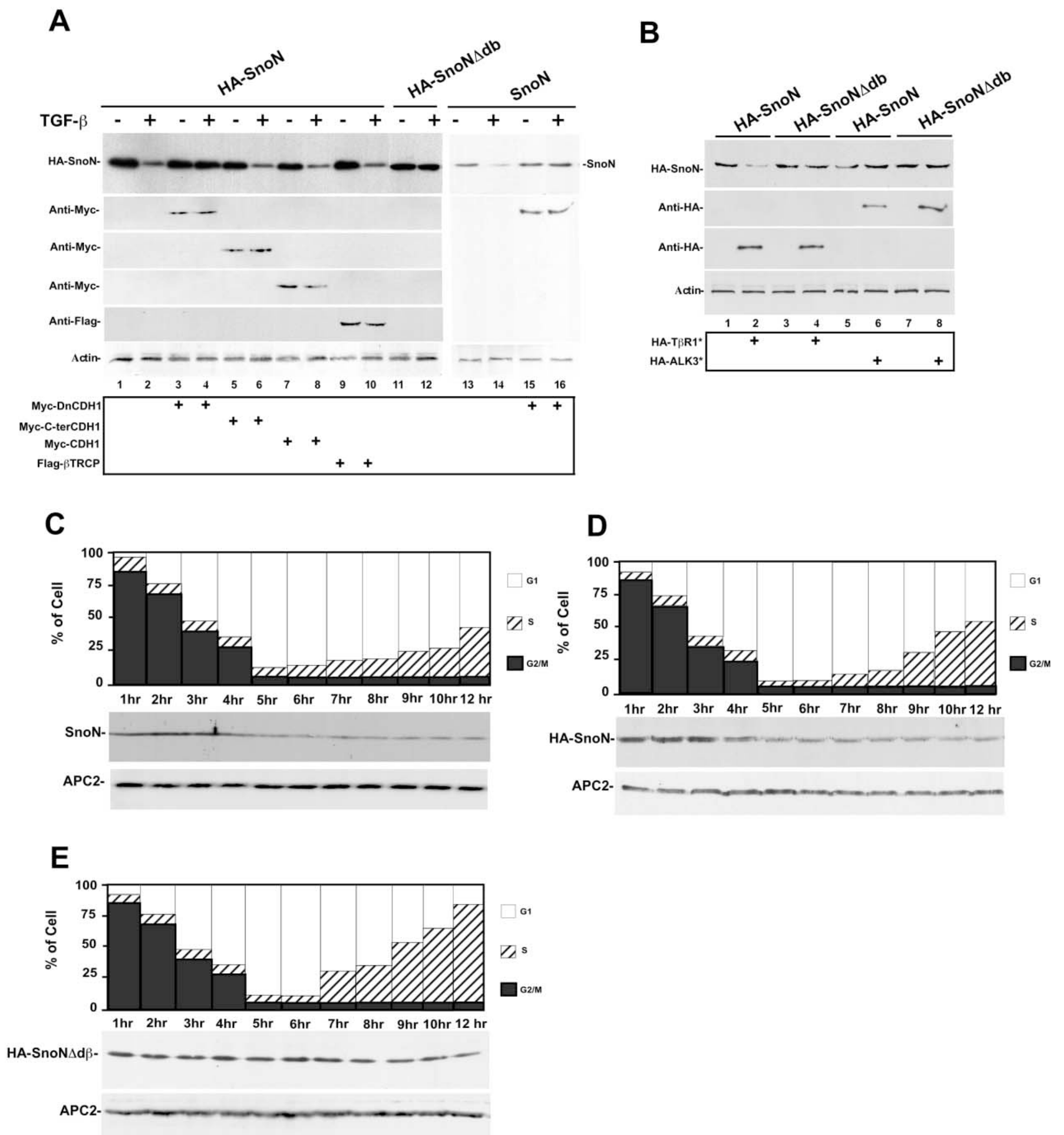

Figure 6. In Vivo Evidence that APC Degrades SnoN

(A) Dominant-negative CDH1 blocks APC-mediated SnoN degradation in TGF-β-stimulated Mv1lu cells. The degradation of exogenous and endogenous SnoN was induced by TGF-β in Mv1lu cells (compare lanes 1 and 2 and lanes 13 and 14). Overexpression of the dominant-negative CDH1 blocked the degradation of both exogenous and endogenous SnoN in response to TGF-β stimulation (compare lanes 3 and 4 and lanes 15 and 16). Overexpression of control constructs, which included carboxy-terminal fragments of CDH1, CDH1, and β-TRCP, did not inhibit SnoN degradation.

(B) Deletion of the destruction box of SnoN attenuates the degradation of SnoN in response to TGF-β signaling followed by activation of TβR1* in EcR293-HA-SnoN/ EcR293-HA-SnoNΔdb stable line cells. SnoN was degraded following activation of TβR1* (compare lanes 1 and 2) but not in the presence of a control receptor (ALK*- constitutively active BMP receptor) (lanes 5–8). Mutation in D box of SnoN attenuated the degradation of SnoN induced by TβR1* (compare lanes 3 and 4).

(C) Cell cycle profile and expression pattern of endogenous SnoN in uninduced EcR293.

(D) Cell cycle profile and expression pattern of exogenous HA-SnoN in induced EcR293-HA-SnoN cells.

(E) Cell cycle profile and expression pattern of exogenous HA-SnoNΔdb in induced EcR293-HA-SnoN cells.

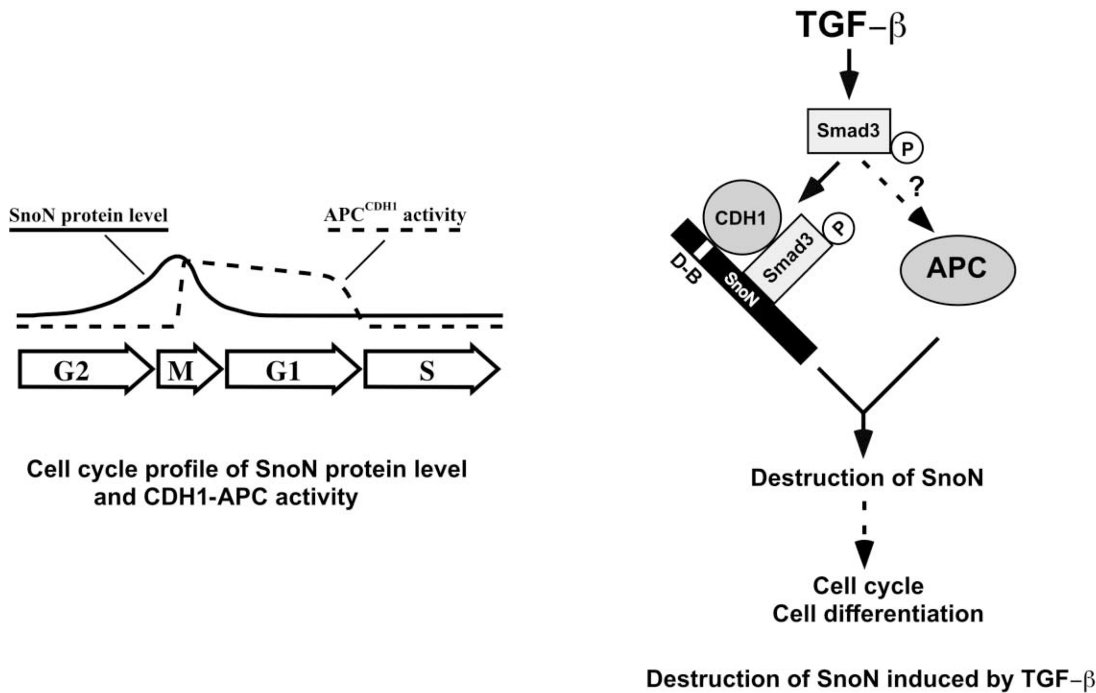

Figure 7. Model for Involvement of APC^{CDH1} in TGF-β Signaling Induced SnoN Destruction

simultaneously, APC, the E3 ligase itself, is a target of regulation (for mitotic cyclins, Securin, and other substrates) rather than the substrates, adding an additional level of potential regulation. Third, a role for APC outside mitosis has been indicated from several experiments, and these results indicate a specific role in G1 by identifying a substrate for APC^{CDH1}. And finally, since much is known about how APC is activated in mitosis, the discovery of its function in another context raises very specific mechanistic questions that can now be addressed.

The cell cycle profile of SnoN is consistent with APC-mediated proteolysis. It accumulates in G2 phase, and it is degraded as cells enter G1. However, in comparison with Cyclin B or CDC20, the degradation of SnoN in G1 is both less precipitous and less complete. Whereas Cyclin B and CDC20 are undetectable in G1, SnoN has merely been reduced to about one quarter of its G2/M levels. The substrate specificity of APC is regulated by two activators of APC; there is partial overlap in specificity between the two activators. CDH1 recognizes substrates with either a D box or a KEN box sequence (Pfleger and Kirschner, 2000), whereas CDC20 recognizes substrates with only a D box sequence (Fang et al., 1998). The *Xenopus* mitotic extracts contain CDC20-activated APC, and they show degradation of SnoN in a D box-specific manner. Similarly, CDH1 will activate an otherwise inactive interphase extract to degrade SnoN in a D box-specific manner. Given the short duration of CDC20-activated APC in the cell cycle (restricted to anaphase/telophase) and the evidence that CDH1-APC will degrade SnoN, we conclude that the G1-specific degradation of SnoN is via CDH1-APC.

To study the detailed mechanism of SnoN degradation under physiological conditions, we developed concentrated somatic cell extracts that show high rates of regulated degradation. In these extracts, Cyclin B can show rates of degradation as fast as those seen in *Xenopus* extracts (half-life 10 min). These somatic extracts, though requiring some care to make, should be very useful for further studies of the regulation of ubiquitin-mediated proteolysis. After brief exposure of cells to TGF-β, the resulting extracts show elevated rates of SnoN degradation. From our experience, there is a real value in measuring aggregate degradation rates rather than ubiquitination rates using purified components, since it is impossible at present to reconstitute all the positive and negative regulators of polyubiquitination at physiological levels.

The increased rate of degradation via APC is not limited to SnoN. Cyclin B and Securin both show acceleration of degradation in extracts made from TGF-β-stimulated cells. The half-life of cyclin B goes from 2 hr to 10 min, the Securin half-life from 6 hr to less than 10 min. This suggests that TGF-β activation may affect a very large range of substrates in addition to SnoN. While this may ultimately be shown to be true, we think it unlikely. The RXXL motif is very common, but based on extensive in vitro expression cloning, we would estimate that only a very small fraction of proteins with this sequence is degraded via APC.

We do not know at present which components of ubiquitination and degradation are affected by the TGF-β pathway. The pathway by which the machinery could be activated is also baffling. There has been very little indication that the TGF-β receptors activate any cytoplasmic events other than those involved in transmitting signals to the nucleus. It has been reported that TGF-β signaling may activate the MAP kinase pathway, but this view is still controversial (Massagué, 1998). All

of the myriad effects of TGF-β are thought to be consequences of the often complex transcriptional networks in which the TGF-β pathways participate. However, the degradation processes occur at too short a time scale for transcription to be important. Activation of APC may turn out to be an important system for investigating the direct cytoplasmic effects of TGF-β signaling.

Smad3 phosphorylation plays an important role in the activation of SnoN degradation in response to TGF-β. Removal of Smad3 from extracts of cells that had previously been exposed to TGF-β prevents the degradation of SnoN. The requirement is specific for phosphorylated Smad3, since only phosphorylated Smad3 can complement the Smad3 depletion. These results are confirmed by coimmunoprecipitation experiments, which detect a ternary complex of CDH1, SnoN, and Smad3. Although complexes between SnoN and CDH1 can be detected in the absence of Smad3, they occur at higher levels if Smad3 is present. Even higher levels of complex formation occur if the cell extracts were derived from cells that had been exposed to TGF-β. Since we know that CDH1 binds the APC, which binds substrates containing D boxes or KEN boxes, these data suggest that Smad3 mediates a quaternary complex of SnoN, CDH1, Smad3, and APC, as shown in Figure 7. We do not know if Smad3 targets a specific APC subunit and therefore increases the affinity of the CDH1-SnoN complex for APC or whether it increases the affinity of CDH1 for SnoN. It might also have some effect on the enzymatic process of polyubiquitination or deubiquitination in addition to its effects on binding.

We investigated directly the sufficiency of CDH1 as an activator of SnoN degradation in extract. Not unexpectedly, CDH1 by itself can have a small effect on SnoN degradation when present at modest concentrations and a large effect at higher concentrations. The effect at low concentrations can be greatly amplified by phosphorylated Smad3. Phosphorylated Smad3 has very little effect on its own, and this effect may be due to residual CDH1 in the extract. These data suggest that unlike Cyclin B, CDC20, and Securin, SnoN is a mediocre substrate for degradation by CDH1 alone, which could account for its small drop in abundance after mitosis. However, it becomes a very good substrate in the presence of phosphorylated Smad3. Phosphorylated Smad3 levels decline after about 8 hr in our experiments, and we see a corresponding decline in the rate of SnoN degradation (Y.W. and M.W.K., unpublished data). Finally we should point out that the experimental tests for synergism between Smad3 and CDH1 did not examine all possible components in the system. Further studies will be required to determine whether CDH1 or components of APC are modified and activated by TGF-β signaling and their quantitative effect on the rate of degradation.

Finally, SnoN levels oscillate in the cell cycle of Hela cells, and this might reveal another implication of the use of APC as the ubiquitin protein ligase. APC is restricted in its activity to a period from metaphase until the G1/S transition by the regulation of its two activators, CDC20 and CDH1. Since CDH1 is an endogenous activator for SnoN degradation, this might limit all TGF-β signaling to the G1 phase of the cell cycle. This could be necessary if the activities of the target genes of the TGF-β pathway are themselves incompatible with processes that occur during the G2 and M phases. For this reason, it would be interesting to know if TGF-β signals can only be transmitted in G1 or in S phase.

Experimental Procedures

Plasmids and Constructs

SnoNΔdb (D box-deleted SnoN) was generated by deleting amino acids 164–172 by a PCR-based approach. The primers used for construction of this mutant are as follows: 5′GTTTCAAGTTGGAG GAGGATTCTCTGTTCTCCGAGAATTTAC3′ and 5′GTAAATTCTCG GAGAACAGAGAATTCTCCTCCAACTTGAAAAC3′. SnoNmdb (D box-mutated SnoN) was generated by replacement of arginine and lucine with alanine at amino acids 164 and 167 by site-directed mutagenesis. The ecdysone-inducible pIND mammalian expression vector, which contains an essential *cis*-acting regulatory element, E/GRE, and a *Drosophila* minimal heat shock promoter (Invitrogen), was used to generate pIND-HA-SnoN and pIND-HA-SnoNΔdb.

Antibodies

Anti-SnoN antibody was obtained from Cascade BioScience. Anti-Myc (A14 or 9E10), anti-HA, anti-β-Actin, anti-Cyclin B, and anti-CDC20 antibodies are from Santa Cruz. Anti-APC2 was raised in our lab previously (Fang et al., 1998).

Preparation of TGF-β-Stimulated Mink Lung Epithelial Cell Extracts

Mink lung epithelial cells (Mv1Lu or ML) were cultured for 24 hr in DMEM medium supplemented with 10% FBS in 5% CO_2. To stimulate cells with TGF-β, they were treated with 100 pM TGF-β (R&D Systems). After 1 hr, cells were washed with PBS and harvested by scraping. Approximately 1×10^8 harvested cells were resuspended in 500 μl hypotonic buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1× protease cocktail (Boehringer Mannheim), and an energy regeneration mixture [Murray, 1991]) for 30 min to allow cells to swell. Cells were frozen by liquid nitrogen, thawed in a 37°C waterbath, and homogenized with ten strokes using a Dounce homogenizer. Cell lysates were spun in an eppendorf microcentrifuge at 14 K rpm at 4°C for 1 hr. The clear supernatant was collected using a syringe needle and used directly for protein degradation assays.

Degradation Assays

For protein degradation assays in *Xenopus* extracts, both interphase and mitotic extracts were prepared as previously described (Fang et al., 1998). ^{35}S-labeled HA-tagged human SnoN protein was synthesized in the TNT expression system (Promega). Approximately 10 ng of IVT-SnoN was added to 20 μl extracts supplemented with degradation cocktail (1.25 mg/ml ubiquitin, 1× energy regeneration, and 0.1 mg/ml cycloheximide). Aliquots were removed at different times and resolved by SDS-PAGE and autoradiography.

For protein degradation assays in Mv1Lu extracts, approximately 10 ng of ^{35}S-labeled SnoN, Cyclin B, or Securin was added to 20 μl fresh Mv1Lu extracts supplemented with the degradation cocktail. Protein degradation was measured as above.

For CDH1 or β-TRCP supplemented experiments, 20 μl extracts were preincubated with approximately 100 ng each of cold IVT CDH1 or β-TRCP proteins, respectively.

Competition Experiments

SnoN degradation assays were performed in the presence of increasing amounts of His-tagged *Xenopus* Cyclin B NH_2-terminal fragment (amino acids 1–102) and NH_2-terminal fragment lacking the D box (CycB-Δdb(N102)) (King et al., 1996).

Coimmunoprecipitation Assays for SnoN and CDH1

Mv1Lu cells were cotransfected with a combination of expression vectors encoding HA-SnoN/Myc-CDH1, HA-SnoN/Myc-CDH1/Flag-Smad3, HA-SnoN/Flag-β-TRCP, and HA-SnoN/Flag-β-TRCP/Flag-Smad3 using Effectin (Qiagen). To assess the interaction of SnoN and CDC20, we also cotransfected Mv1Lu cells with combinations of expression vectors encoding HA-SnoN/Myc-CDC20 and HA-SnoN/

Myc-CDC20/Flag-Smad3. The transfected cells were preincubated with proteasomal inhibitor MG-132 (50 μM) or vehicle (DMSO) for 1 hr before addition of TGF-β. HA-SnoN complexes were pulled down by anti-HA matrix (Roche). Interaction between SnoN and CDH1, β-TRCP, or CDC20 was judged by protein immunoblotting with anti-Myc (Santa Cruz) or anti-Flag antibodies (Sigma).

Purification of Activated Smad3

Approximately 200 ng cold IVT Smad3 was added to 50 μl Mv1Lu extracts that had either been prepared from cells exposed to TGF-β or not. The extracts were incubated at room temperature for 60 min. Activated Smad3 was subsequently purified by addition of 5 μl anti-Flag matrix (Sigma). After 2 hr incubation, beads were spun and extensively washed. Immunopurified Smad3 was used for the rescue experiments.

Immunodepletion of Smad3

Anti-Smad3 antibodies (Santa Cruz) were bound to protein A-conjugated Dynabeads 280 (Dynal) and incubated for 1 hr on a rotating wheel. Beads were washed once with PBS, 0.1% Triton X-100, once with 0.5 M NaCl in PBS, 0.1% Triton X-100, and three times with XB (10 mM HEPES [pH 7.7], 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 5 mM EGTA) (Murray, 1991). Beads were retrieved on a magnet and used for depletion. Extracts were depleted with addition of Smad3-conjugated beads and incubated on ice for 1 hr with several gentle mixes by pipetting. Mock depletions were performed in the same way except that normal rabbit serum was used instead of anti-Smad3. All extracts were depleted twice and then used for degradation assays.

Purification of His-CDH1 and GST-Smad3 Proteins

His-tagged hCDH1 was expressed in insect cells by using Baculovirus (Fang et al., 1998). Sf9 cells were infected with the virus and harvested 50 hr later. Protein was purified over a nickel column and eluted with 250 mM imidazole. The purified protein was dialyzed and added to 15% glycerol before freezing in liquid nitrogen.

GST-tagged Smad3 was expressed in *E. coli* strain BL21. Expression was induced with IPTG, and the protein was purified over a glutathione-agarose column and eluted with reduced glutathione. The fractions were collected and dialyzed against XB buffer. Glycerol was added to 15%, and then the fraction was frozen in liquid nitrogen and stored at −80°C.

Development of HA-SnoN and HA-SnoNΔdb Stable Cell Lines

EcR-293 cells, which stably express the ecdysone receptor, were purchased from Invitrogen and maitained in DMEM medium plus 400 μg/ml of Zeocin (Invitrogen). The EcR293-HA-SnoN and EcR293-HA-SnoNΔdb cell lines were created by transfecting EcR 293 with 25 ug of pIND-HA-SnoN or pIND-HA-SnoNΔdb by using the calcium phosphate method. Stable transfectants were selected for 4 weeks in DMEM plus 200 μg/ml of Hygromycin and 400 μg/ml Zeocin. Surviving clones were individually cloned from the stable pool and expanded. For induction of HA-SnoN and HA-SnoNΔdb expression, 2 μg/ml of ponasterone-A (Invitrogen) was added to the cell cultures. Clones were screened for HA tag expression after 12 hr induction and selected for further analysis.

Synchronization of Hela S3, HA-SnoN, and HA-SnoNΔdb HEK293 Cells and Cell Cycle Analysis

Hela S3 cells were arrested at the G1/S boundary by growth in the presence of 2 mM thymidine (Sigma) for 18 hr followed by washing with PBS and by growth in fresh medium without thymidine for 8 hr. Finally, thymidine was added again to 2 mM to block cells at G1/S; after another 18 hr, cells were transferred to fresh medium, and samples were harvested every hour up to 12 hr.

To arrest EcR293-HA-SnoN or EcR293-HA-SnoNΔdb cells in mitosis, cells were first treated with 2 mM thymidine, released into fresh medium for 4 hr, and then blocked in medium containing 100 ng/ml nocodazole for 12 hr. For release from mitosis, cells were washed with PBS and transferred into fresh medium; samples were collected every hour up to 12 hr.

For flow cytometry, synchronized cells were harvested at set time points, fixed in 70% ethanol, washed in PBS, and then treated with 100 U/ml of RNase A and stained with propidium iodide. At least 30,000 cells were scored using FACS flow cytometry. Cell cycle distribution was analyzed with CellQuest software.

Cell Sorting

To isolate Mv1Lu cells expressing ectopic SnoN, dominant-negative CDH1 and GFP or EcR293-HA-SnoN/EcR293-HA-SnoNΔdb cells transiently expressing constitutively active TGF-β type I receptor (TβR1*) or control receptor ALK3* (constitutively active BMP receptor) and GFP, were stimulated with TGF-β (30 min) (only for Mv1Lu cells) 30 hr after transfection by Effectin method cells and sorted according to their GFP levels by a FACSVantage cell sorter (Becton-Dickinson).

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