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Proteasomal activity modulates TGF-ß signaling in a gene-specific manner

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Abstract Transforming growth factor-β (TGF-β) signaling relies on Smad-signaling pathway controlled in part by the proteasome. Here we demonstrate that inhibition of the proteasome function in mink epithelial cells accumulates both positive and negative modulators of TGF-β signaling, phospho-Smad2 and SnoN. Inhibition of the proteasome led to abrogation of TGF-β target gene regulation in a gene-specific manner. While regulation of *p15Ink4b* and *myc* by TGF-β are lost, *PAI-1* induction, previously shown to occur in a Smad3-dependent manner, was not affected by treatment of the cells with the proteasomal inhibitor MG132. The results suggest that proteasomal activity is required for TGF-β signaling in a gene-specific manner.

Key words: Transforming growth factor-β; Smad; SnoN; Proteasome; Signaling

1. Introduction

Transforming growth factor-β (TGF-β) signaling is initiated by binding of the ligand to type I and II receptor complex, which activates the phosphorylation of signal transducers Smad2 and Smad3 [1,2]. After phosphorylation, Smad2 and Smad3 recruit a co-Smad, Smad4, to form heteromeric complexes and migrate to the nucleus, where they either bind directly to DNA or associate with other transcription factors to regulate transcription of a large number of genes including *myc*, *junB* and *p15Ink4b* cdk inhibitor (for review see [1,3]).

Several key transducers in the TGF-β-signaling pathway are affected by the ubiquitin–proteasome system. Proteasome pathway is involved in destruction of Smad2 and Smad3 whose degradation switches off TGF-β signaling [4–7]. Conversely, inhibitory Smad7, after binding to TGF-β receptors is also subject to proteasomal degradation [8]. Interestingly, SnoN (Ski-related novel protein N), a newly identified repressor of TGF-β signal transduction pathway ([9,10], reviewed in [11]), is also degraded via the ubiquitin–proteasome pathway, mediated by anaphase–promoting complex (APC) or Smurf2 E3 ligases [12–14]. SnoN functions like a 'switch' in TGF-β signal transduction: it interacts directly with the Smad2/3–Smad4 complexes and represses their transactivation abilities, thus shutting off the signaling [15–17]. The capacity to inhibit Smad2/3 signaling is shared by Ski, a SnoN-homolog and prototype of the Ski-family of oncogenes ([16], for review see [11]). The dissociation of SnoN from the Smads and its degradation via the ubiquitin pathway allows the signaling to pass through. Accordingly, TGF-β signal transduction is controlled by the proteasome pathway both positively and negatively: degradation of SnoN maintains the signal while degradation of phosphorylated Smads turns it off. Regulation between these two events ensures TGF-β signaling to continue or stop at appropriate times corresponding to the environmental cues of the cells. Considering the importance of the proteasome-mediated degradation of TGF-β signaling molecules, it is relevant to address the question whether TGF-β signaling is maintained if the proteasome function is abrogated. Here, we inhibit the function of the ubiquitin–proteasome pathway and demonstrate that the TGF-β-mediated early signaling is intact in vivo in Mv1Lu mink lung epithelial cells, but is abrogated at later timepoints in a gene-specific manner apparently through an increase of SnoN.

2. Materials and methods

2.1. Cells, cell culture and reagents

Mv1Lu mink lung epithelial cells (CCL64, American Type Cultured Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and were kept at 5% CO_2 atmosphere at 37°C. In all assays, the cells were treated with 100 pM of TGF-β or 10 μM of MG132 (MG, Z-Leu-Leu-Leu-CHO, Affinity Research Products Ltd., Mamhead, UK) in DMEM containing 10% FCS. TGF-β was purified from outdated human platelets.

2.2. Antibodies

SnoN antibody used for immunoblotting and immunofluorescence (H-317) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and phospho-Smad2 antibody was a kind gift from Peter ten Dijke and Carl-Henrik Heldin [18]. Smad2/3 antibody (610843) was from BD Transduction Laboratories. Biotin-conjugated goat anti-rabbit, goat anti-mouse and streptavidin antibodies were from DAKO (Glostrup, Denmark). Goat anti-rabbit Alexa 594 or 488 were from Molecular Probes.

2.3. Immunoblotting

Cells were lysed with 50 mM Tris–HCl buffer, pH 6.8, containing 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), and 10% glycerol and DNA was sheared by sonication as described before [19]. Total cellular proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore, Bradford, MA, USA). Immunoblotting with the indicated antibodies was carried out as described before [20] followed by detection with enhanced chemiluminescence (Amersham Life Sciences). Multi-Analyst program 1.0.2 (Bio-Rad) was used to quantitate the signals.

2.4. Immunofluorescence staining

Cells grown on glass coverslips were fixed with 3.5% paraformal-

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dehyde in PBS for 20 min at room temperature and then permeabilized with 0.5% Nonidet P-40 for 5 min. Coverslips were incubated with anti-SnoN or anti-phospho-Smad2 antibody at 37°C for 1 h followed by incubation with either anti-rabbit Alexa 594 or 488 at 37°C for another 1 h. Nuclei were counterstained with Hoechst 33258 (Sigma). The staining was visualized with Axioplan 2 MOT microscope (Zeiss, Jena, Germany) equipped with appropriate filters.

2.5. Northern analysis

Poly A+-RNA was isolated with GenElute™ Direct mRNA miniprep kit (Sigma, St. Louis, MO, USA). mRNA (2 μg) was separated on 1% agarose–formaldehyde gel and transferred to Hybond-N^+ membrane in 20×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate). mRNA was detected by probing with human *p15INK4B*, *myc*, *junB*, *PAI-1* or *GAPDH* fragments labeled with (γ-^{32}P)dCTP by random priming (Ready-to-Go, Pharmacia).

3. Results and discussion

3.1. Inhibition of the proteasome augments the levels of phospho-Smad2

Since both positive and negative regulators of the TGF-β-signaling pathway are controlled by ubiquitin-mediated degradation, we wanted to analyze the cellular levels of Smad2 and SnoN, the primary effectors of TGF-β signaling. Smad2 is rapidly phosphorylated (phospho-Smad2) following the ligand binding initiating the intracellular signaling cascade. The phosphorylation is subsequently required for its degradation through the proteasome pathway [1]. We treated Mv1Lu epithelial cells with physiologically relevant concentrations of TGF-β known to elicit a growth inhibitory response within 12–16 h and analyzed the levels of phospho-Smad2 with or without treatment of MG132, a specific proteasomal inhibitor that blocks the chymotryptic activity of the 26S proteasome. TGF-β induced a rapid, 10-fold increase in the levels of phospho-Smad2 as detected by immunoblotting (Fig. 1A,B) and immunofluorescence analyses (not shown). Increased levels of phospho-Smad2 still persisted at a late timepoint (16 h) though they had declined from the highest level of induction observed at 4 h (Fig. 1A,B). Treatment of Mv1Lu cells with MG132 gradually increased the phospho-Smad2 levels, and by 16 h its level was increased by nine-fold (Fig. 1A,B). Combination of TGF-β and MG132 during the treatment increased the levels of phospho-Smad2 over that of TGF-β treatment alone, and showed a marked increase at 16 h (Fig. 1A,B). A similar change is visualized by immunofluorescence showing a clear accumulation of phospho-Smad2 in nucleus in TGF-β- and MG132-treated cells, indicating that TGF-β signal transduction is intact in this respect (Fig. 3). Total levels of Smad2/3 increased by two-fold as shown for the 16 h timepoint in Fig. 1A.

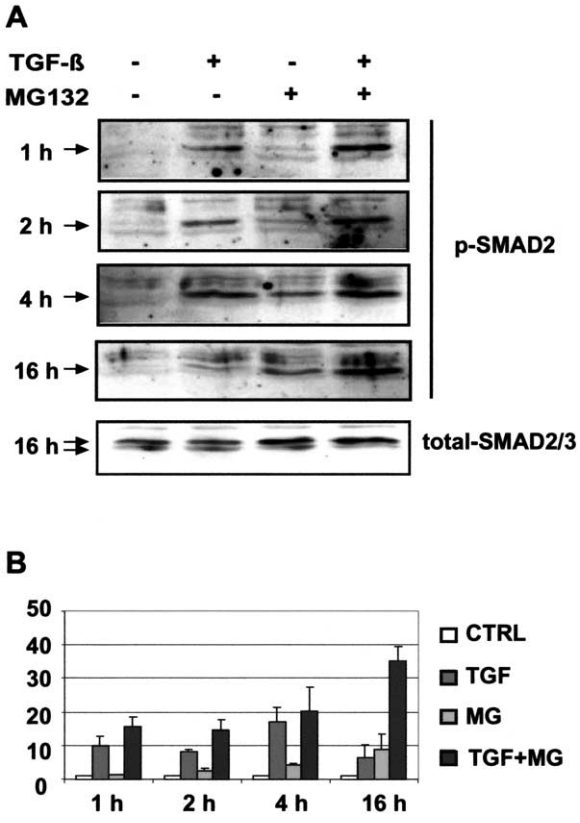

Fig. 1. MG132 modulates TGF-β effects on phospho-Smad2. Mv1Lu cells were treated with TGF-β (100 pM) and MG132 (10 μM) as indicated for the times shown. Total cell extracts were prepared and phospho-Smad2 protein levels were analyzed by immunoblotting using an phospho-Smad2/3 antibody or an antibody detecting total Smad2/3 (A). The signals were quantitated and the average of two independent experiments with standard error are shown (B). The amount of phospho-Smad2 in control cells was set as 1. Ctrl, control; MG, MG132; p-Smad2, phospho-Smad2.

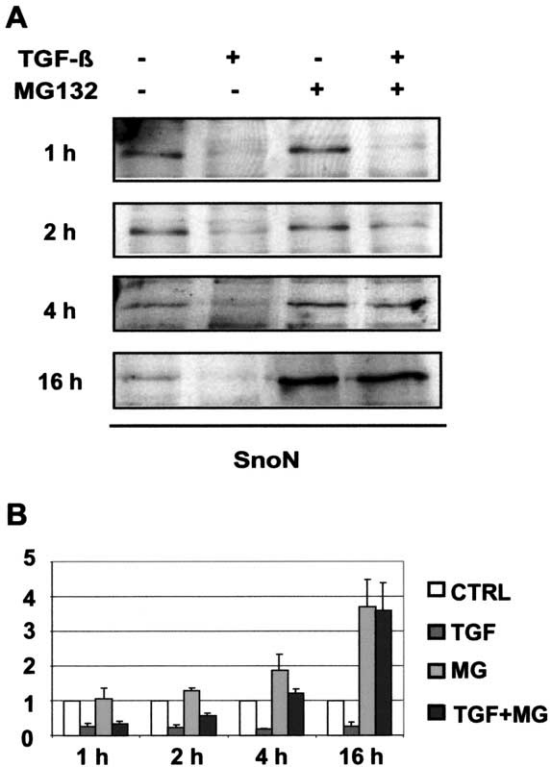

Fig. 2. MG132 increases SnoN levels and prevents TGF-β-mediated downregulation. Total cell extracts of Mv1Lu cells treated with TGF-β and MG132 as indicated were prepared and SnoN protein levels were analyzed by immunoblotting (A) followed by quantitation of the signals (B). The results are the average of two independent experiments with standard error shown. Ctrl, control; MG, MG132.

3.2. MG132 increases SnoN and prevents TGF-β-mediated downregulation at a late timepoint

Next, we analyzed the levels of SnoN, which is degraded

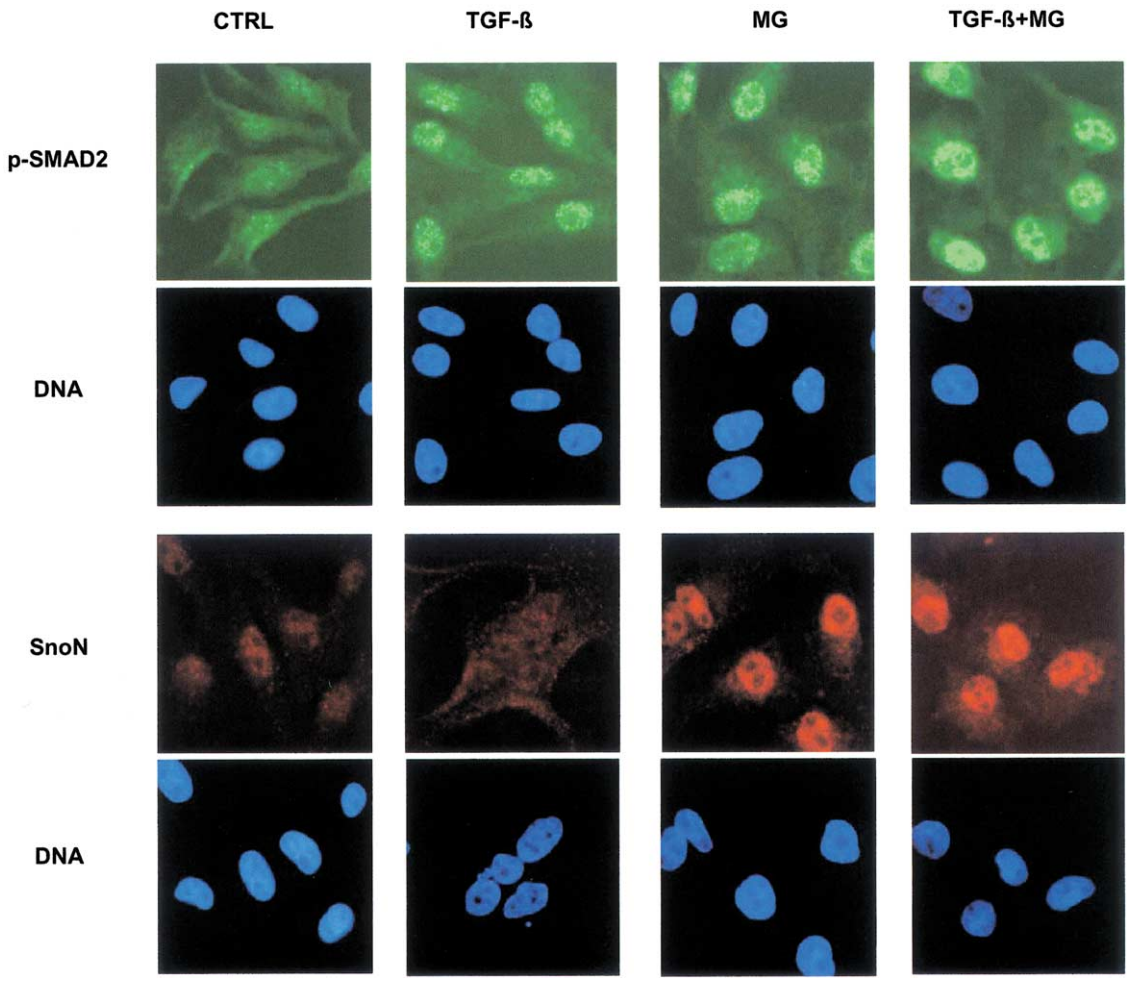

Fig. 3. MG132 increases the levels of phospho-Smad2 and SnoN. Mv1Lu cells were treated with TGF-β and MG132 for 16 h followed by immunofluorescence analysis for phospho-Smad2 and SnoN. DNA was stained by Hoechst 33258. A representative result of three independent experiments is shown. Ctrl, control; MG, MG132.

through the proteasome in both Smad-dependent and -independent manner [9,12–14]. As described previously [9], TGF-β rapidly reduced SnoN protein level within 1 h and SnoN was maintained low throughout the follow-up as shown both by immunoblotting and immunofluorescence (Figs. 2A,B and 3). Treatment of the cells by MG132 caused a progressive increase in SnoN levels up to 16 h, indicating that it is undergoing proteolytic degradation also in the absence of TGF-β signaling (Figs. 2A,B and 3; refs. [9,14]). MG132-treatment of the cells did not prevent the initial decrease of SnoN by TGF-β within 2 h, but completely blocked the TGF-β-mediated decrease of SnoN observed 16 h after addition of TGF-β (Figs. 2A,B and 3). This suggests that MG132 treatment has relatively little effect on TGF-β signaling components early on after its addition, whereas at later timepoints it possibly can abrogate TGF-β signaling by inhibiting SnoN downregulation.

Earlier reports on regulation of SnoN by TGF-β have indicated that the rapid decrease of SnoN is mediated through proteasomal degradation [12–14,16], but that longer TGF-β treatments increase SnoN levels through an increase of SnoN mRNA [10]. Transcriptional induction of a signaling repressor is reminiscent to that of p53 induction of its E3-ubiquitin ligase and thus repressor, Mdm2, generating a succession of rapid oscillations in the levels of p53 and mdm2 [21]. Thus when signaling is permissive, i.e. levels of SnoN are low, an increase in SnoN and its binding to Smad2/3 will switch off the signal until SnoN is degraded below a threshold level. Analysis of endogenous phospho-Smad2 and SnoN, as demonstrated here, indicated that abrogation of the proteasome activity appears mainly to affect late (16 h) TGF-β-regulated events by maintaining elevated levels of phospho-Smad2 as well as SnoN. Therefore it was pertinent to address whether TGF-β signaling is functional under these conditions.

3.3. *Downregulation of the proteasome allows early TGF-β signaling but abrogates late TGF-β effects in a target-gene-specific manner*

We carried out Northern analyses of genes regulated by the TGF-β–Smad pathway, *myc* [22], *junB* [23], *PAI-1* [24] and *p15* [25]. Of these, *junB* and *PAI-1* are rapid early response genes for TGF-β [26–28], *p15* is induced with slower kinetics [29] and *c-myc* is downregulated by TGF-β [30]. The results showed that TGF-β induction of *junB* and *p15* was intact 2 h after addition of MG132 (three- and 1.5-fold inductions, respectively), but *p15* mRNA was somewhat diminished by 4 h (Fig. 4A,B). Though initial induction of *PAI-1* in the presence of MG132 was lower than by TGF-β alone (24 vs. 12-fold),

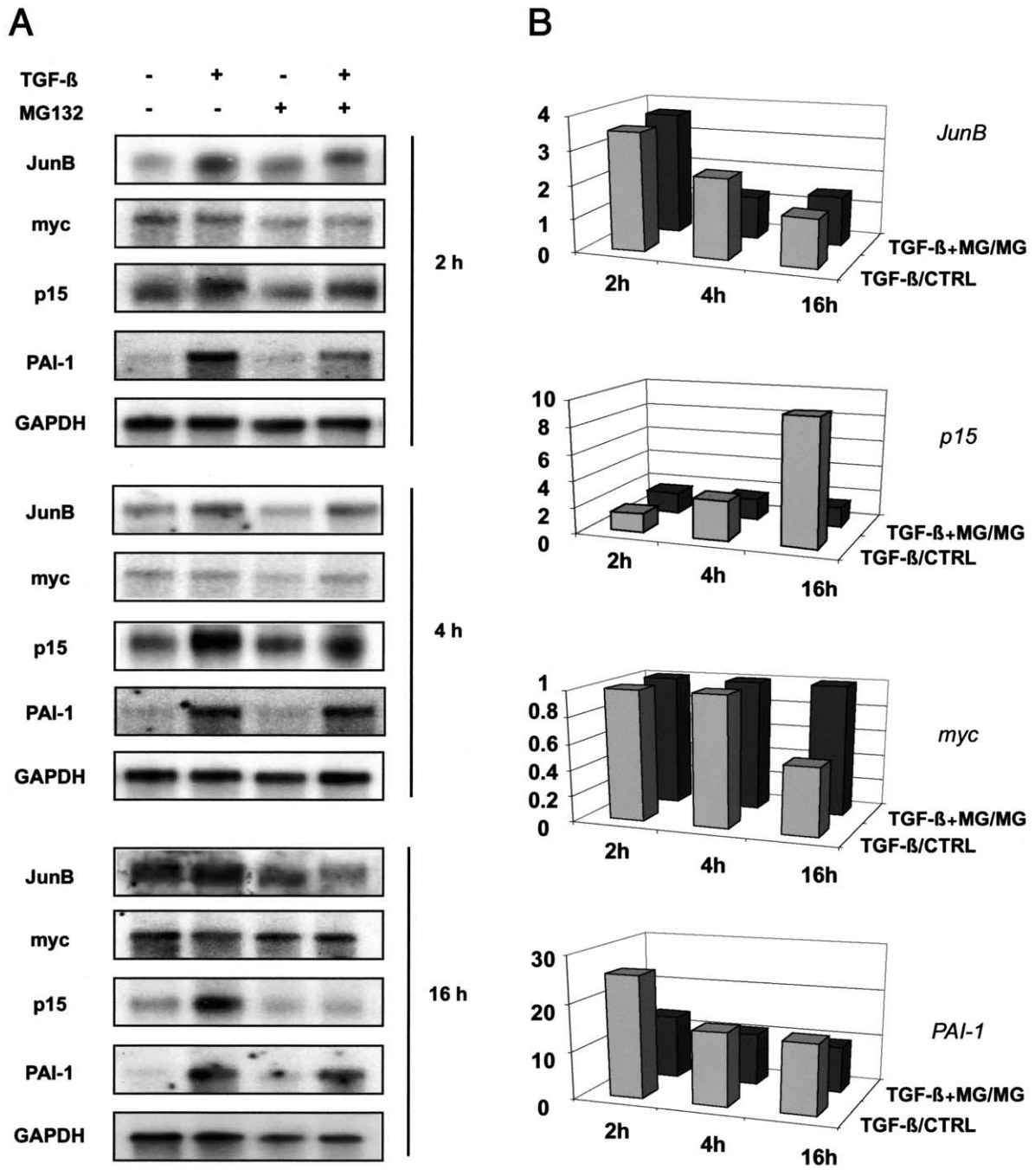

Fig. 4. TGF-β regulation of gene responses is modulated by proteasome inhibition. Mv1Lu cells were treated with TGF-β and MG132 as indicated for the times shown and the regulation of the indicated genes was analyzed by Northern assays (A). GAPDH was used as loading control. The signals were quantitated by Multi-Analyst and normalized against GAPDH levels. Relative levels on the basis of three independent experiments are shown (B).

the inductions were comparable by 4 h (Fig. 4A,B). However, MG132 abrogated TGF-β-mediated induction *p15* by 16 h, whereas *PAI-1* induction was maintained (Fig. 4). Due to the nature of *junB* being an early response gene, its induction by TGF-β was transient and no longer detectable at the late timepoint. Downregulation of *myc* by TGF-β was observed at late timepoints and this effect was completely abrogated by MG132 (Fig. 4). The results indicate that treatment of the cells with the proteasomal inhibitor allows substantial TGF-β signaling to pass through at least initially, but that the signaling is disrupted late after inhibition of the proteasome function in a target-gene-specific manner.

The regulation of TGF-β target genes via Smad-signaling pathway can utilize either Smad2 or Smad3-dependent events or poses a requirement for the presence of both Smads. Piek et al. [31] show by using Smad2 or -3 null cells, that while TGF-β induction of *p15* requires both Smad2 and Smad3, *PAI-1* and *junB* require only Smad3. Accordingly, our observation that downregulation of the proteasome will allow TGF-β induction of *PAI-1*, but not *p15*, is suggestive that

Smad3 pathway is intact, whereas that of Smad2-dependent pathway is abrogated by MG132. Smad2 is degraded through the proteasome in a Smurf2-dependent manner [5,7], and Smad3 through ROC1 [32]. SnoN, on the other hand, is targeted to the proteasome both by Smad2–Smurf2 complex and by Smad3–APC complex. Therefore, an increase in SnoN levels is projected to block both Smad2 and Smad3-dependent signaling depending on the relative ratio of the proteins. Though Smad7–TGF-β receptor complex is also degraded by the proteasome [8], a possible increase in Smad7 is unlikely to inhibit Smad2-related signaling as high levels of phospho-Smad2 are still detected in the nucleus.

In response to proteasome inhibition we observe significant increases in both phospho-Smad2 as well as SnoN. A potential selectivity of SnoN towards either Smad2 or Smad3 may arise from differences in the relative levels of the respective proteins or other factors determining their interactions and thus the ability of SnoN to repress Smad-dependent transcription. The block of TGF-β signaling towards only specific target genes suggests that proteasomal activity is required selectively in the TGF-β-signaling pathway.

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