

Transforming Growth Factor- β /SMAD Target Gene *SKIL* Is Negatively Regulated by the Transcriptional Cofactor Complex SNON-SMAD4^{*[5]}

Received for publication, May 29, 2012; Published, JBC Papers in Press, June 6, 2012; DOI 10.1074/jbc.M112.386599

Angeles C. Tecalco-Cruz¹, Marcela Sosa-Garrocho, Genaro Vázquez-Victorio, Layla Ortiz-García, Elisa Domínguez-Hüttinger, and Marina Macías-Silva²

From the Departamento de Biología Celular y Desarrollo, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, Distrito Federal 04510, México

Background: Human *SKIL* gene encodes for SNON, a negative regulator of the TGF- β /SMAD pathway.

Results: We provide a molecular mechanism of transcriptional regulation of *SKIL* gene expression by TGF- β /SMADs.

Conclusion: Transcriptional cofactor complex SNON-SMAD4 negatively controls the expression of *SKIL* gene.

Significance: The formation and function of complex SNON-SMAD4 are impaired in cancer cells lacking SMAD4, which affects TGF β -target gene regulation.

The human *SKI*-like (*SKIL*) gene encodes the SMAD transcriptional corepressor SNON that antagonizes TGF- β signaling. SNON protein levels are tightly regulated by the TGF- β pathway: whereas a short stimulation with TGF- β decreases SNON levels by its degradation via the proteasome, longer TGF- β treatment increases SNON levels by inducing *SKIL* gene expression. Here, we investigated the molecular mechanisms involved in the self-regulation of *SKIL* gene expression by SNON. Bioinformatics analysis showed that the human *SKIL* gene proximal promoter contains a TGF- β response element (TRE) bearing four groups of SMAD-binding elements that are also conserved in mouse. Two regions of 408 and 648 bp of the human *SKIL* gene (~2.4 kb upstream of the ATG initiation codon) containing the core promoter, transcription start site, and the TRE were cloned for functional analysis. Binding of SMAD and SNON proteins to the TRE region of the *SKIL* gene promoter after TGF- β treatment was demonstrated by ChIP and sequential ChIP assays. Interestingly, the SNON-SMAD4 complex negatively regulated basal *SKIL* gene expression through binding the promoter and recruiting histone deacetylases. In response to TGF- β signal, SNON is removed from the *SKIL* gene promoter, and then the activated SMAD complexes bind the promoter to induce *SKIL* gene expression. Subsequently, the up-regulated SNON protein in complex with SMAD4 represses its own expression as part of the negative feedback loop regulating the TGF- β pathway. Accordingly, when the SNON-SMAD4 complex is absent as in some cancer cells lacking SMAD4 the regulation of some TGF- β target genes is modified.

TGF- β cytokine regulates several cellular processes such as proliferation, differentiation, and apoptosis mainly through the activation of SMAD transcriptional factors (1–4). Because of the variety of SMAD2 (S2),³ SMAD3 (S3), and Co-SMAD4 (S4) heteromeric complexes that can be generated, the transcription of most TGF- β target genes can be differentially regulated in a cell context-dependent manner (4). In addition, many of the TGF- β /SMAD actions can be antagonized by nuclear *SKI* and *SKI*-novel (SNON) proteins, which are two closely related members of the *SKI* family of oncoproteins that were identified by their homology with the viral transforming protein v-*SKI* (5–8). SNON and *SKI* act as SMAD corepressors by interacting with SMAD complexes to inhibit their transcriptional abilities and by recruiting other corepressors and histone deacetylases (HDACs) to diverse TGF- β -responsive gene promoters (5, 9–11). So far, only a few TGF- β target genes have been shown to be directly regulated by *SKI* and SNON. The *SMAD7* gene, a negative regulator of the TGF- β pathway, is likely the best characterized gene negatively regulated by *SKI* and SNON corepressors (12, 13). In addition, SNON and *SKI* proteins can also be localized in the cytosol where they might be able to block TGF- β signals by sequestering SMAD proteins and preventing their translocation to the nucleus (5, 14).

TGF- β tightly regulates SNON and *SKI* protein stability by inducing their degradation via the ubiquitin-proteasome system (UPS) in a SMAD-dependent manner (15–22). Interestingly, the TGF- β /SMAD pathway regulates SNON protein levels in a biphasic manner: it causes a rapid and transient SNON protein degradation via the proteasome followed by an up-regulation of SNON mRNA and protein levels after a longer TGF- β treatment. This newly synthesized SNON protein seems to establish a negative feedback loop to turn off TGF- β signaling;

^{*} This work was supported in part by Universidad Nacional Autónoma de México/Dirección General Asuntos del Personal Académico/Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica Program Grants IN222909 and IN206012 and Consejo Nacional de Ciencia y Tecnología (CONACyT) Grants 49493-Q and 101826.

^[5] This article contains supplemental Figs. S1–S5 and Tables S1–S3. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) JX103164.

¹ Recipient of a Ph.D. fellowship from CONACyT.

² To whom correspondence should be addressed. Tel.: 52-55-56-22-5729; Fax: 52-55-56-22-5611; E-mail: mmacias@ifc.unam.mx.

³ The abbreviations used are: S2, SMAD2; IP, immunoprecipitation; S3, SMAD3; S4, SMAD4; SBE, SMAD-binding element; SNON, *SKI*-novel; TRE, TGF- β response element; UPS, ubiquitin-proteasome system; WB, Western blot; *SKIL*, *SKI*-like; TSS, transcription start site; HDAC, histone deacetylase; re-ChIP, sequential ChIP; TSA, trichostatin; NaB, sodium butyrate; ANISO, anisomycin; R-SMAD, receptor-regulated SMAD.

this is an important but poorly understood event (13, 23). The regulation of SNON expression is relevant because SNON has an essential role during embryonic development as well as in homeostasis in the adult organism. SNON is expressed at low levels in embryonic and postnatal tissues, but its expression can be increased in some tissues at specific stages of embryonic development or in different physiological contexts (5, 6). Moreover, SNON protein up-regulation may have a relevant role in regulating the magnitude and duration of TGF- β signaling.

SKIL knock-out causes lethality in mice because *Sno* gene is required for blastocyst formation (5, 6). *Sno* +/− mice with very low SNON protein expression are more susceptible to tumorigenesis, suggesting a tumor suppressor role for SNON (5, 6, 24). In contrast, high levels of *SNON* mRNA and protein have been reported in many cancer cell types, also suggesting an oncogenic role. In fact, the overexpression of SNON seems to contribute to cell resistance to TGF- β -induced growth arrest in some cell types and also induces anchorage-independent growth of chicken and quail embryo fibroblasts (10, 14, 24–27). Therefore, it has been proposed that high levels of SNON protein might lead to tumor growth, whereas low levels may lead to tumor metastasis (26). Interestingly, *SNON* mRNA and protein levels are up-regulated during liver regeneration, a model of cell proliferation distinct from cancer where SNON probably functions in restraining the mitoinhibitory effect of the TGF- β /SMAD pathway (28); in contrast, low levels of SNON have been observed in renal fibrosis where it might favor TGF- β profibrotic actions (29, 30).

It is clear that a tight regulation of SNON expression is critical for SNON to function adequately in space and time. However, the transcriptional regulation of *skil* gene is not completely understood. Recently, the mouse *Sno* (*SKIL*) gene promoter was cloned and partially characterized in fibroblasts (25). This promoter bears a TGF- β response region with four SMAD-binding element (SBE) groups that bind S2-S4 complexes to activate *SKIL* gene expression. The promoter also contains a SMAD inhibitory element downstream of the SBE region that binds the inhibitory S3-S4 complex, and that study clearly demonstrated that mouse *SKIL* gene is a TGF- β /SMAD pathway target. Intriguingly, the authors also observed a prolonged induction of SNON expression by TGF- β , which was shown to have a critical role in fibroblast transformation (25).

Here, we cloned and analyzed a fragment of the human *SKIL* gene promoter bearing the core and proximal promoter as well as a TGF- β response element (TRE) containing several SBEs. We focused on studying the molecular mechanisms involved in the self-regulation of *SKIL* gene expression by SNON protein in different cell contexts.

EXPERIMENTAL PROCEDURES

Bioinformatics Analysis—A genomic DNA sequence of ~5 kb located immediately upstream from the ATG of human *SKIL* gene was obtained from GenBank™ (accession number AC073288). This sequence was analyzed to predict the putative promoter region, transcription factor binding sites, and TSS of human *SKIL* gene by using several software tools including GenBank, FPRO, FirstEF, DBTSS, GPminer, and ALGGEN-PROMO (supplemental Table S1 and Fig. S1).

Cloning of the Human *SKIL* Gene Promoter Bearing TRE and Generation of Plasmid Constructs—Two fragments of the *SKIL* gene harboring the promoter (408 and 648 bp) were amplified by PCR from human genomic DNA (obtained from freshly isolated human blood leukocytes) using AccuPrime GC-rich DNA polymerase (Invitrogen) and specific primers flanked by KpnI and SacI restriction sites. These *SKIL* gene fragments are located at positions −3100/−2692 (408 bp) and −3100/−2451 (648 bp) upstream from ATG (+1). The reporter plasmids were constructed by cloning each fragment into KpnI and SacI sites of pGL3-Basic vector (Promega) to obtain the reporter genes skilSBEs(408)-Luc (408-bp fragment) and skilSBEs(648)-Luc (648-bp fragment). The cloned 408- and 648-bp fragments of the *SKIL* promoter contained all four SBE groups and the TSS (+1). The *SKIL* promoter region of 408 bp was also cloned with an inverted orientation in the pGL3-Basic vector by subcloning the insert into KpnI and XhoI sites of pcDNA3.1 and then subcloned into HindIII and XhoI sites of pGL3-Basic to obtain the reporter skilSBEs(408, 3′–5′)-Luc. The region of 408 bp was also subcloned into KpnI and SacI sites of pGL3 minimal promoter-E1B-Luc to obtain the reporter skilSBEs(408)-E1B-Luc. All constructs were sequence-verified.

Cell Lines—A549 (human lung carcinoma) cells were maintained in Ham's F-12 medium, and SW480 (human colon carcinoma) cells were cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (1:1), whereas HepG2 (human hepatoma) and AD293 (a clone derived from HEK293 human embryonic kidney cell line) cells were maintained in DMEM. Growth medium was supplemented with 10% fetal bovine serum (FBS) plus antibiotics (penicillin/streptomycin), and cells were maintained under a 5% CO₂ atmosphere at 37 °C. AD293 and A549 cells stably expressing pRS/shSnoN (catalog number TR309425 from OriGene), pRetroSuper/shSmad4J hygro (Addgene plasmid 19151), or pBABE/Smad4J Rescue (Addgene plasmid 19153) (31) were maintained in the presence of 10 μ g/ml puromycin or 200 μ g/ml hygromycin as selection antibiotics.

SNON Site-directed Mutagenesis—DmSNON(Δ S2/S3/S4) and UBmSNON(K437A,K446A) were generated by site-directed mutagenesis on the pCIneo/HA-SnoN (wild type mouse SNON) using specific primers (supplemental Table S3) according to the manufacturer's instructions (Stratagene). All constructs were sequence-verified.

Luciferase Assays—For TGF- β -inducible luciferase assays, A549, HepG2, SW480, and AD293 cells were transiently transfected with the reporter plasmids containing fragments of the *SKIL* gene promoter and pCMV/ β -gal with or without any of the following plasmids: pCMV5/T β RI-HA (wild type (WT)), pCMV5/T β RI-HA (T204D), pCMV5/T β RI-HA (K232R), pCMV5/FLAG-Smad2, pCMV5/FLAG-Smad3, pCMV5/HA-Smad4, pCIneo/HA-Ski, pCIneo/HA-SnoN, pCIneo/HA-Dm-SnoN, or pCIneo/HA-UBmSnoN. Cells were seeded at 60% confluence in 12-well plates and transiently transfected with 0.5–1 μ g of total DNA/well using the Lipofectamine method for A549 cells or calcium phosphate method for SW480 and AD293 cells as described previously (3, 13). 24 h posttransfection, cells were treated for 12 h with 100 pM TGF- β 1 (Prepro-Tech), then they were lysed, and luciferase activity (Promega)

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was measured in a luminometer (Turner Biosystems). β -Galactosidase activity was used to normalize for transfection efficiency.

RT-PCR Assay—SNON and β -actin mRNA levels were detected by RT-PCR using the primers and conditions described previously (13). In brief, total RNA was isolated using TRIzol (Invitrogen) from cells treated with or without 300 pM TGF- β for the indicated times. Total RNA (2 μ g) was used for cDNA synthesis using random hexamers and Moloney murine leukemia virus RT (Invitrogen), and PCR was carried out using Taq PCR Master Mix kit (Qiagen) using specific primers (supplemental Table S2). PCR products were analyzed by electrophoresis on agarose gels.

Immunoprecipitation and Western Blot—Cells were lysed with TNTE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA containing 0.5% Triton X-100 plus a mixture of protease and phosphatase inhibitors) as described previously (13). Proteins were immunoprecipitated with specific anti-SMAD2/SMAD3 (N-20), anti-SMAD4 (C-20), anti-SNON (H-317) polyclonal antibodies (Santa Cruz Biotechnology) or rabbit polyclonal anti-SMAD4 (Upstate/Millipore), and then proteins were separated by SDS-PAGE and detected by immunoblotting with specific primary antibodies and either anti-rabbit or anti-goat secondary HRP-conjugated antibody (Zymed Laboratories Inc.) by using an enhanced chemiluminescence assay (ECL kit from Amersham Biosciences). Phospho-SMAD2 was detected with a specific polyclonal antibody (Chemicon/Millipore).

Chromatin Immunoprecipitation (ChIP) and Sequential ChIP (Re-ChIP) Assays—ChIP assays were carried out as described previously (13) with the following modifications. Cells were treated with 1% formaldehyde at 37 °C for 15 min and then sonicated on ice for 10 cycles of 30 s each with a Fisher Sonic Dismembrator 300. The desired amount of protein-cross-linked DNA extract was precleared in batches, and specific antibodies were used for immunoprecipitation. Oligonucleotide sequences used for PCR were the same used for cloning the human SKIL gene promoter (supplemental Table S2). PCR products were analyzed by electrophoresis on agarose gels. For re-ChIP, the DNA-protein complexes immunoprecipitated with the indicated antibodies were eluted with 10 mM dithiothreitol, diluted 20 \times in re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0), and then reimmunoprecipitated with the indicated antibodies (32).

ChIP on Reporter Plasmid—AD293 cells were transiently transfected with 3 μ g of skilSBEs(408)-Luc reporter plasmid by the Lipofectamine method. Cells were cross-linked with formaldehyde 48 h post-transfection and harvested, and then a ChIP assay was performed as described previously (33). The obtained DNA fragments were analyzed by PCR with specific primers to amplify the region of SKIL promoter on the reporter (supplemental Table S2).

Northern Blot—Total RNA was purified from either primary cultured mouse hepatocytes or the human HepG2 cell line using TRIzol (Invitrogen). Northern blots were performed as described previously (28).

Wound Healing Assay—Confluent A549 cells were serum-starved for 12 h, and then a wound was generated across the cell monolayer using a 1-mm plastic tip. Cell pictures were taken at

different time points (0, 24, 48, and 72 h) after wounding. Data were expressed as a percentage of wound closure.

Statistical Analysis—A Student's *t* test was used to calculate statistical significance. A *p* value <0.05 was considered to be significant.

RESULTS

SNON Expression Is Regulated by TGF- β at the Transcriptional Level—SNON is a regulatory protein capable of antagonizing TGF- β /SMAD signaling. Several studies have shown that TGF- β tightly regulates the expression levels of SNON protein and mRNA (13, 15–23, 25, 28, 34, 35). Northern blot analysis using total RNA from HepG2 cells or mouse hepatocytes showed that SNON mRNA was induced after 1-h treatment with 300 pM TGF- β . We detected three main mRNA transcripts for human SKIL gene (~6.2, 3.5, and 3.0 kb) and for mouse Skil gene (~6.2, 3.6, and 3.2 kb) (Fig. 1A). The main difference in the size of these transcripts is probably due to different lengths of their 3'-UTRs as has been reported previously (34, 35). In fact, the largest transcript reported in GenBank (accession number NM_005414.4) contains two poly(A) sites in the 3'-UTR (supplemental Fig. S1). Furthermore, the cycloheximide pretreatment induced an accumulation of SNON mRNA transcripts in both HepG2 cells (Fig. 1A) and mouse hepatocytes (data not shown). These results confirm that TGF- β positively regulates SNON expression at the transcriptional level, and it does not require *de novo* protein synthesis.

Interestingly, the analysis of SNON and SNON2 isoform expression showed that human cell lines such as HepG2 and A549 mainly expressed SNON protein isoform, which was also the main isoform induced by TGF- β treatment (Fig. 1B). In contrast, in mouse cell line C2C12 (Fig. 1B) and mouse hepatocytes (data not shown), both SNON and SNON2 protein isoforms were expressed, although SNON2 was the most abundant isoform expressed and the main isoform induced by TGF- β .

To better understand the transcriptional regulation of human SKIL gene, we first performed a bioinformatics analysis of an ~5-kb genomic DNA sequence from human chromosome 3, which is located immediately upstream of the ATG of SKIL gene (GenBank accession number AC073288). We used the prediction programs GPminer, Genomatix, ALGGEN-PROMO, DBTSS, FPRO, and FirstEF (supplemental Table S1) to determine the promoter region of SKIL gene including the TSS. We also performed an alignment between a human chromosome 3 clone (GenBank accession number AC073288) and the ~7.2-kb clone of human SNON mRNA (accession number NM_005414.4) using NCBI-BLAST and Aspic. Results revealed that the promoter of SKIL gene including a region with some potential TSSs was located at ~2.6 kb upstream from the ATG. The analysis showed that the promoter region of SKIL gene has a high G+C content with GC boxes, but neither TATAA nor CAAT boxes were found. Also, we detected a CpG island (~1.6 kb) spanning the promoter of SKIL gene by using the CpG Island Searcher and EMBOSS programs (supplemental Fig. S1C and Table S1).

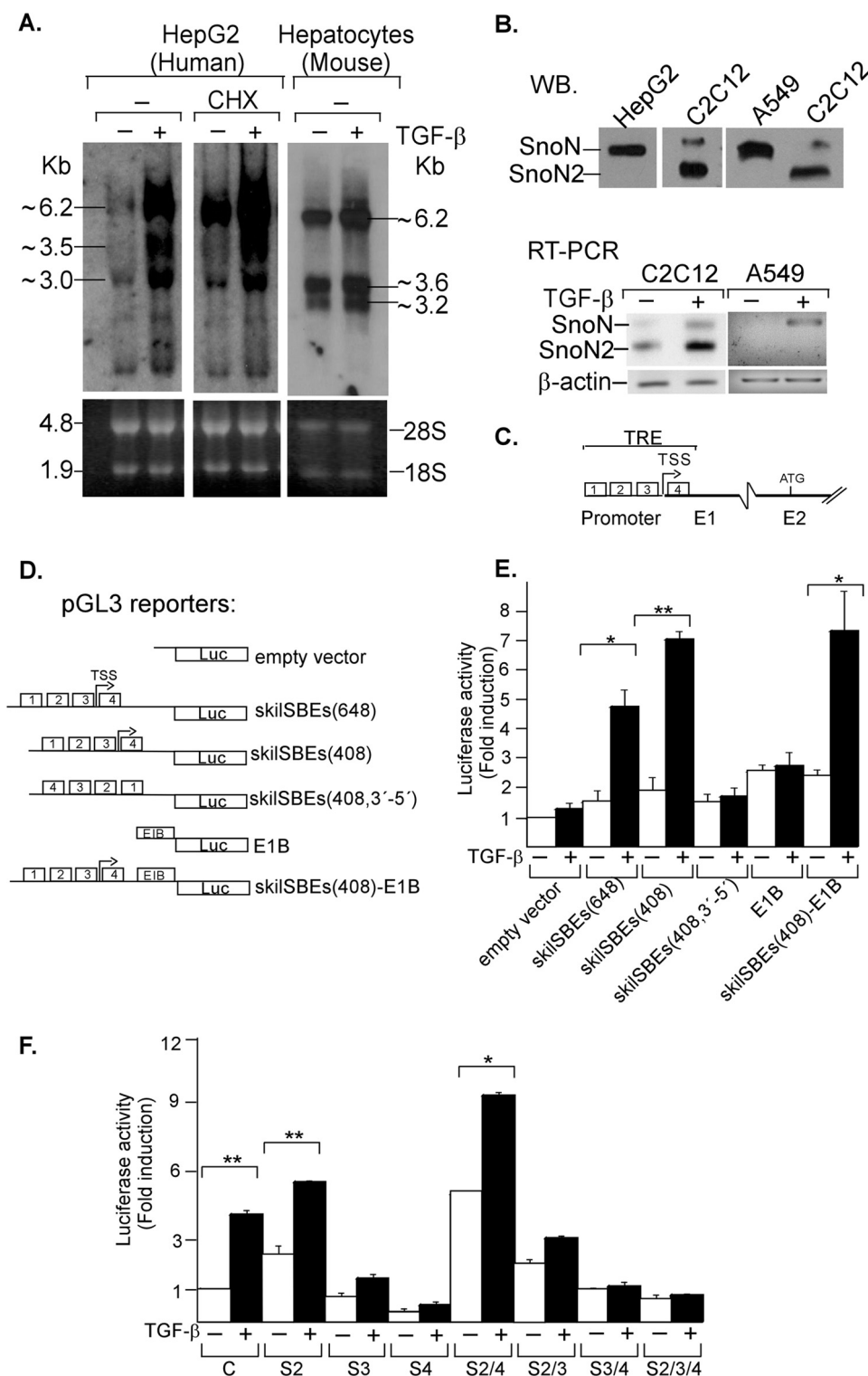


FIGURE 1. TGF- β regulates SNON expression at the transcriptional level. *A*, TGF- β induces the early expression of three main SNON mRNA transcripts. HepG2 cells and mouse hepatocytes were treated for 1 h with 300 pM TGF- β . HepG2 cells were also pretreated in the absence or presence of 20 μ g/ml cycloheximide (CHX) for 20 min. Total RNA was isolated, and Northern blot analysis of SNON mRNA was performed. rRNAs (18 and 28 S) are shown as an RNA loading control. *B*, to analyze SNON and SNON2 protein expression, whole cell protein extracts from human HepG2 or mouse C2C12 cells were immunoprecipitated and then detected by WB with specific anti-SNON antibodies (upper panel), or total RNA was obtained from human HepG2 or mouse C2C12 cells treated for 1 h with or without 300 pM TGF- β to analyze SNON and SNON2 mRNA levels by RT-PCR (lower panel). *C*, the assembled SKIL gene map shows the localization of promoter, TRE, putative TSS, ATG (+1), exon 1 (E1), and exon 2 (E2). *D*, the pGL3 reporter gene constructs bearing different fragments of the SKIL gene promoter or empty vectors are schematically shown. *E*, AD293 cells transiently transfected with the indicated reporter plasmids were incubated for 12 h in the absence or presence of 100 pM TGF- β , and cell extracts were analyzed for luciferase activity. *F*, AD293 cells were transfected with the skiSBEs(408)-E1B-Luc reporter along with plasmids bearing full-length cDNA for S2, S3, or S4, and then cells were incubated for 12 h in the absence or presence of 100 pM TGF- β . Cells were lysed, and samples were analyzed for luciferase activity. Luciferase activity was normalized using β -gal expression and expressed as -fold induction over control. Values are mean \pm S.E. (error bars) of three separate experiments in triplicate. *, $p < 0.05$; **, $p < 0.01$ compared with control (C).

Further analysis showed that a ~450-bp sequence spanning the core promoter of the *SKIL* gene has four groups of putative binding sites for SMAD transcription factors (SBEs) (supplemental Figs. S1, S2, and S3); this region corresponds to the TRE that is conserved in the mouse *Skil* gene promoter (supplemental Figs. S1, S2, and S3). These SBEs found in the human *SKIL* promoter showed high identity to those previously identified on the mouse *Skil* promoter by footprinting (25). Each one of the four SBEs in the *SKIL* promoter has one or two consensus sites for SMAD binding that were identified previously as important regulatory motifs for the expression of the mouse *Skil* gene controlled by TGF- β (25).

On the other hand, the analysis of the 5'-region of *SKIL* gene structure showed the presence of a small first exon (~170 bp) followed by the first intron (1933 bp) and part of the second exon (633 kb) (supplemental Fig. S1A). Thus, the *SKIL* gene contains seven exons, the first exon is noncoding, the ATG is localized at 5'-half of the second exon (supplemental Fig. S1A), and the size of the main *SNON* mRNA transcript generated is ~7.2 kb (supplemental Fig. S1B). The position of the putative TSS (+1) of *SKIL* gene was localized between SBE3 and SBE4 of TRE from data obtained via partial cloning of the 5'-UTR by RT-PCR assay (supplemental Fig. S2, A, B, and C). The 5'-UTR fragment of *SNON* mRNA (839 bp) was cloned and sequence-verified (supplemental Fig. S2D). This partial nucleotide sequence of the 5'-UTR (from -2738 to -2571 and from -635 to +37 nucleotides) was submitted to GenBank under accession number JX103164 (supplemental Fig. S3).

In addition, we found several consensus sites for different transcription factors on this region by bioinformatics analysis (supplemental Fig. S4). Some of these transcription factors are known SMAD partners such as CCAAT/enhancer-binding proteins, p53, NF κ B, STATs, MYOD, and YY1 among others (11). These data support that this region contains a functional gene promoter that is probably regulated by different signaling pathways under specific cellular contexts or in cross-talk with the TGF- β pathway.

The TGF- β -responsive Region of the *SKIL* Gene Promoter Is Also SMAD-responsive—To study the regulation of the TRE region of the *SKIL* promoter by TGF- β , we made two main reporter plasmids that include the SBE region and TSS, skilSBEs(408)-Luc and skilSBEs(648)-Luc, each one bearing a fragment of 408 or 648 bp, respectively (Fig. 1, C and D). These reporter gene constructs were transiently transfected into AD293 cells; pGL3-Basic vector was used as a control. As expected, both reporters were clearly responsive to the TGF- β stimulus (Fig. 1E). Thus, our results indicate that the region of the human *SKIL* gene promoter that contains the SBEs is part of the proximal promoter of *SKIL* gene and is TGF- β responsive. We also obtained similar results with a reporter plasmid bearing the mouse *Skil* gene promoter (data not shown).

We also observed that the transcriptional activity of the *SKIL* gene promoter (skilSBEs(408)-Luc) is specific and orientation-dependent because the same sequence cloned in an inverted orientation into pGL3-Basic (skilSBEs(408, 3'-5')-Luc) lost TGF- β -induced activity (Fig. 1E). We made another reporter gene named skilSBEs(408)-E1B-Luc, which contains the minimal E1B promoter and the 408-bp fragment of the *SKIL* pro-

motor; this reporter had a response to TGF- β similar to that of the reporter lacking the E1B promoter (Fig. 1E). These results support the conclusion that the region corresponding to the promoter of *SKIL* gene is spanned by the TRE.

We used the skilSBEs(408)-Luc reporter construct to characterize the response to TGF- β ; thus, different epithelial cell lines were transiently transfected with this reporter, and its activity was measured by a luciferase assay. This reporter was responsive to TGF- β stimulus in all the different cell lines tested such as AD293, A549, and HepG2 (data not shown). We observed that the induction of this reporter was also dependent on TGF- β concentration in all cells tested (data not shown). In addition, the constitutively active form of the TGF- β receptor ALK5 (T204D mutation) was able to induce the *SKIL* gene promoter, whereas the WT ALK5 and a kinase-deficient ALK5 (K232R mutation) were inactive in AD293 cells (data not shown). Moreover, a pretreatment with the ALK5 inhibitor SB431542 (10 μ M) (Tocris), which specifically blocks SMAD2 and SMAD3 phosphorylation, clearly prevented *SKIL* gene promoter induction by TGF- β in AD293 cells (data not shown).

We then analyzed the activation of *SKIL* gene promoter by overexpressing different SMAD proteins in AD293 cells. The S2 or S2-S4 overexpression increased *SKIL* gene promoter expression and also enhanced the induction by TGF- β , whereas S3, S4, S2-S3, S3-S4, or S2-S3-S4 overexpression showed an inhibitory effect (Fig. 1F). Thus, TGF- β positively regulates *SKIL* promoter activity mainly through specific S2-S4 complexes. Our results agree with data reported previously for the mouse *Skil* promoter (25).

SMAD and SNON Proteins Bind to and Regulate *SKIL* Gene Promoter—To examine whether SNON was able to negatively regulate its own expression, we performed luciferase assays by transiently co-transfecting AD293 cells with the skilSBEs(408)-Luc reporter along with plasmids bearing WT HA-Ski or HA-SnoN cDNAs. We observed that SNON and SKI were potent inhibitors of *SKIL* gene promoter activity (Fig. 2A) and that the *SKIL* promoter expression inhibited by SNON was dependent on the concentration of the transfected pCneo/HA-SnoN plasmid (Fig. 2B).

Several studies have proposed that SNON and SKI can associate with some repressor factors that interact with HDACs to inhibit gene expression (10, 13, 36–39). To understand how SNON inhibits the transcriptional expression of its own gene, we evaluated the effect of HDAC inhibitors trichostatin (TSA) and sodium butyrate (NaB). *SNON* mRNA levels were analyzed in A549 cells pretreated for 1 h with or without 0.1 μ M TSA or 5 mM NaB and then incubated in the presence or absence of 300 pM TGF- β for 2 h. Data showed that HDAC inhibition by TSA or NaB increased basal *SNON* mRNA levels and facilitated induction of *SKIL* gene by TGF- β (Fig. 2C). These inhibitors also increased the basal and TGF- β -induced activity of skilSBEs(408)-Luc gene reporter (Fig. 2, D, E, and F). The S2-S4 complexes increased skilSBEs(408)-Luc reporter activity, and this was favored by TSA treatment (Fig. 2D). Interestingly, HDAC inhibitors also blocked the repression of skilSBEs(408)-Luc reporter caused by SNON protein (Fig. 2, E and F). Data showed that SNON self-represses its gene by forming a repressor complex with HDAC activity.

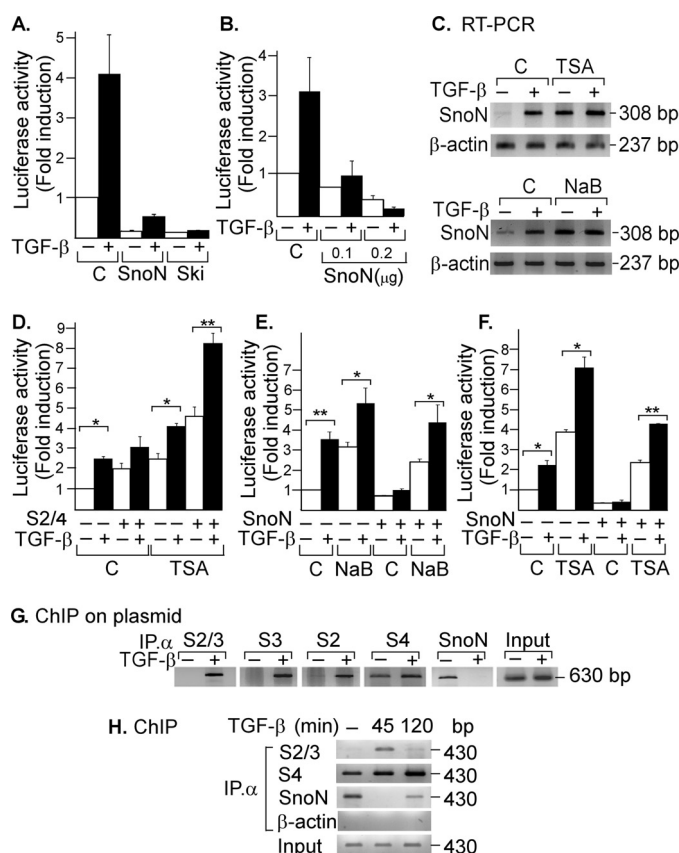


FIGURE 2. SNON binds to the *SKIL* promoter and represses its own expression. AD293 cells were transiently transfected with skiSBEs(408)-Luc along with HA-Ski or HA-SnoN cDNAs (A) or with different amounts of pCneo/HA-SnoN (B). Cells were incubated for 12 h in the absence or presence of 100 pM TGF- β , then lysed, and analyzed for luciferase activity. Luciferase activity was normalized using β -gal expression and is reported as -fold induction over control. Values are expressed as means \pm S.E. (error bars) of three separate experiments in triplicate. C, A549 cells were pretreated for 30 min with or without the HDAC inhibitors TSA (100 nM) (upper panel) and NaB (5 mM) (lower panel) and then incubated for 2 h in the absence or presence of 300 pM TGF- β . Total RNA was isolated, and SNON (308-bp) and β -actin (237-bp) mRNAs were amplified by RT-PCR with specific primers ($n = 3$). D, AD293 cells transfected with skiSBEs(408)-Luc reporter along with plasmids bearing cDNAs for S2 and S4 were pretreated for 30 min with or without 100 nM TSA and then incubated for 12 h in the absence or presence of 100 pM TGF- β . E and F, AD293 cells transfected with skiSBEs(408)-Luc reporter with or without HA-SnoN were pretreated for 30 min with or without 5 mM NaB or 100 nM TSA and then incubated for 12 h in the absence or presence of 100 pM TGF- β , and then cells were lysed and analyzed for luciferase activity. Luciferase activity was normalized using β -gal expression and is reported as -fold induction over control. Values are expressed as means \pm S.E. (error bars) of three separate experiments in triplicate. *, $p < 0.05$; **, $p < 0.01$ compared with control (C). G, AD293 cells were transiently transfected with skiSBEs(408)-Luc reporter, and 48 h post-transfection, cells were incubated for 45 min in the absence or presence of 500 pM TGF- β ($n = 2$). H, A549 cells were incubated for 45 min or 2 h in the absence or presence of 500 pM TGF- β ($n = 2$). ChIP on plasmid (G) or ChIP (H) assays were carried out using anti-S2/S3, anti-S2, anti-S3, anti-S4, or anti-SNON antibody. PCRs were done with primers spanning the SBE region either from the pGL3 vector bearing the promoter fragment (630 bp) or a region from the endogenous *SKIL* promoter (430 bp).

Expression of the *SKIL* gene is positively regulated by SMADs and negatively regulated by SNON. Thus, we evaluated the binding of endogenous SMAD and SNON proteins to the *SKIL* gene promoter by ChIP on a plasmid assay. We first analyzed the binding of endogenous S2, S3, and S4 proteins on skiSBEs(408)-Luc reporter plasmid previously transfected along with HA-SnoN cDNA in AD293 cells. Cell extracts were immunoprecipitated with specific antibodies for anti-S2/S3,

-S2, -S3, -S4, or -SNON, whereas anti- β -actin antibody was used as a control, and the DNA that co-immunoprecipitated with these proteins was used to amplify the region of the pGL3 vector bearing the *SKIL* promoter fragment (630 bp) by PCR. It was observed that endogenous S2 and S3 proteins interacted with the SBE region only in response to TGF- β , whereas SNON was bound to that region only at the basal state. Intriguingly, S4 was bound to the *SKIL* promoter in basal conditions, and the binding was further increased by TGF- β stimulation (Fig. 2G). These results demonstrate that SNON and SMAD proteins bind differentially to the human *SKIL* gene promoter and indicate that the repression and activation of the *SKIL* gene promoter occur at different time points during TGF- β signaling.

We further evaluated the dynamic of endogenous SMAD and SNON binding to the *SKIL* gene promoter in response to TGF- β , considering that TGF- β exerts a fine-tuned time-dependent regulation of SNON levels. We performed ChIP assays using A549 cells treated with 500 pM TGF- β for different time points (0, 45, and 120 min). Immunoprecipitations were carried out with anti-S2/S3 and anti-SNON specific antibodies, and then the co-immunoprecipitated DNA was amplified by PCR with specific primers for the *SKIL* gene promoter (430 bp). Data showed that the endogenous activated S2 and S3 were transiently bound to the *SKIL* gene promoter after TGF- β treatment (45 min), and then their binding decreased at 120 min (Fig. 2H). In contrast, we observed that endogenous SNON protein was associated with the *SKIL* gene promoter at the basal level, but this association decreased shortly after TGF- β treatment, which coincides with SNON down-regulation induced by TGF- β . Interestingly, after a longer TGF- β treatment (>2 h) when SNON protein levels were up-regulated, the SNON protein was observed to bind back to the *SKIL* promoter to repress it probably as part of a negative feedback loop generated by TGF- β itself (Fig. 2H). This result demonstrates that SNON and SMADs bind to the *SKIL* gene promoter and also provides a molecular mechanism for the temporal repression and activation of *SKIL* gene promoter during TGF- β /SMAD signaling.

The Expression of SNON Is Regulated at Different Levels—SNON expression is regulated at multiple levels such as gene transcription, mRNA stability, and translation as well as at the level of protein stability. For this reason, it is difficult to find the correlation between SNON mRNA and protein levels at specific time points after TGF- β stimulus. Therefore, to analyze the time course of induction of SNON mRNA in response to TGF- β , A549 cells were incubated for different times with or without 300 pM TGF- β . Total RNA was then isolated, and a fragment (308 bp) of SNON mRNA and a fragment (317 bp) of SMAD7 mRNA were amplified by RT-PCR with specific primers (Fig. 3, A and B). Analyzing the time course of induction of SNON, we observed that SNON mRNA levels were increased 2 h after stimulus and remained elevated until 4 h later (Fig. 3A). We observed that 2 h after TGF- β stimulation the SNON-S4 repressor complex was again positioned on the *SKIL* promoter instead of the S2-S4 activator complex; thus, under this scenario, we investigated why the levels of SNON mRNA remained elevated for so long.

To evaluate whether the levels of SNON mRNA remained elevated due to an increase in its stability or to continuous *SKIL*

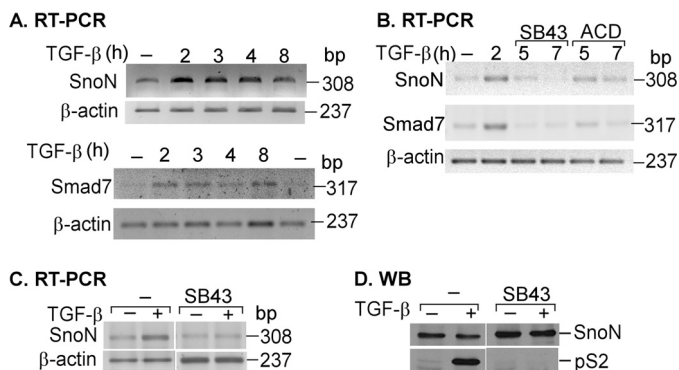


FIGURE 3. SNON mRNA and protein levels are regulated at different levels after TGF- β stimulus. A, A549 cells were incubated for different times with or without 300 pM TGF- β . Total RNA was isolated, and then *SNON* (308-bp), *SMAD7* (317-bp), and β -actin (237-bp) mRNAs were amplified by RT-PCR with specific primers ($n = 3$). B, A549 cells were incubated for different times in the presence or absence of 300 pM TGF- β , and in the indicated cases, 5 μ g/ml actinomycin D (ACD) or 10 μ M SB431542 (SB43) was added 2 h post-treatment with TGF- β . Total RNA was isolated, and RT-PCR was performed with specific primers for *SNON* (308 bp), *SMAD7* (317 bp), and β -actin (237 bp) ($n = 2$). C and D, A549 cells were preincubated for 30 min in the presence or absence of 10 μ M SB431542 and then incubated for 1 h with or without 300 pM TGF- β . Total RNA was isolated, and RT-PCR was performed with specific primers for *SNON* and β -actin ($n = 2$) (C), or whole cell protein extracts were immunoprecipitated with anti-SNON or anti-S2/S3 antibody and then detected by WB with anti-SNON or anti-phospho-S2 antibody ($n = 3$) (D).

gene transcription, we used actinomycin D to inhibit transcription and SB431542 to inhibit SMAD activation. A549 cells were incubated for 2 h in the presence or absence of 300 pM TGF- β , and then 5 μ g/ml actinomycin D or 10 μ M SB431542 was added for different times (Fig. 3B). Total RNA was isolated, and RT-PCR was performed with specific primers for *SNON*. We observed that SB431542 induced a faster decrease of *SNON* mRNA levels than actinomycin D, suggesting that activated SMADs may play a role not only in inducing *SKIL* gene transcription but also in controlling *SNON* mRNA stability (Fig. 3B). Similar results were obtained when *SMAD7* mRNA levels were analyzed (Fig. 3, A and B); *SMAD7* was identified previously as a *SNON* target gene (13).

Because multiple factors are involved in controlling *SNON* expression, it has been difficult to observe a correlation between *SNON* mRNA and protein levels. Thus, we studied the effect of SB431542 on *SNON* expression. Serum-starved A549 cells were preincubated for 30 min in the absence or presence of 10 μ M SB431542 and then incubated for 2 h with or without 300 pM TGF- β . Total RNA was isolated, and RT-PCR was performed with specific primers for *SNON* (Fig. 3C), or whole cell protein extracts were immunoprecipitated with anti-SNON or anti-S2/S3 antibody and then detected by immunoblot with anti-SNON or anti-phospho-S2 (Fig. 3D). Data showed that SB431542 treatment decreased *SNON* mRNA levels but increased *SNON* protein levels at the basal conditions. Thus, the inactivation of SMADs seems to promote low levels of *SNON* mRNA and high levels of *SNON* protein, suggesting a role for SMADs in controlling both *SNON* mRNA and protein stability.

SNON Is Removed from *SKIL* Gene Promoter upon TGF- β Stimulation Independently of Its Degradation—It has been proposed that *SNON* and *SKI* corepressors maintain some TGF- β target genes repressed in the absence of ligand; however, only a

few *SKI* and *SNON* target genes have been identified so far. Upon TGF- β stimulation, S2 and S3 translocate into the nucleus and induce a rapid degradation of *SNON* and *SKI* proteins via the proteasome. Thus, it has been argued that *SNON* and *SKI* degradation induced by TGF- β is necessary to allow the activation of different TGF- β target genes, but the exact underlying mechanisms have not been clearly determined. TGF- β /SMAD signaling induces *SNON* protein degradation via the UPS involving E3 ubiquitin ligases such as Arkadia, SMURF2, or anaphase-promoting complex; activated R-SMADs participate as adapters that recruit the E3 ubiquitin ligases required for the polyubiquitination of *SKI* and *SNON* (15–22). Previously, we reported that the antibiotic anisomycin (ANISO) can also down-regulate *SNON* and *SKI* proteins via the proteasome but through a new mechanism that is independent of SMAD proteins and from its known ribotoxic effects, and now anisomycin has become a useful tool to down-regulate *SKI* and *SNON* levels in specific cell types (16, 40).

We set up an assay to evaluate whether *SNON* degradation induced by TGF- β was required to induce *SKIL* gene expression. A549 cells were preincubated for 2 h with or without 50 μ M MG132, a specific proteasomal inhibitor, and then cells were incubated for 45 min in the absence or presence of 300 pM TGF- β or 10 μ M ANISO. TGF- β and ANISO decreased *SNON* protein levels in the absence of a proteasome inhibitor, whereas treatment with MG132 prevented *SNON* down-regulation but not S2 phosphorylation (Fig. 4A). We also observed that *SNON* protein levels were increased over basal in A549 cells preincubated with MG132 likely because of greater protein stability, whereas the basal mRNA levels did not change significantly. However, *SNON* mRNA levels were induced by TGF- β with or without MG132 pretreatment, but the induction was lower in cells pretreated with MG132 (Fig. 4B). These data indicated that TGF- β and ANISO reduce *SNON* protein levels by the UPS as shown previously (13, 16).

MG132 pretreatment showed a slight inhibitory effect on TGF- β actions such as the increase of *SNON* mRNA levels (Fig. 4B), *SNON* binding to *SKIL* gene promoter (Fig. 4C), and activation of *SKIL* gene promoter (Fig. 4D). In the latter case, MG132 pretreatment decreased basal *skil*SBEs-Luc reporter gene expression, whereas the TGF- β -induced expression of the *SKIL* promoter was only slightly affected (Fig. 4D). These data suggest that TGF- β promotes *SNON* protein down-regulation to fully induce *SKIL* gene expression. To test this, we used a *SNON* mutant (UBmSNON) unable to be polyubiquitinated and degraded (17). We observed that UBmSNON repressed *skil*SBEs-Luc reporter activity in a similar way as WT *SNON* (Fig. 4E). We also performed ChIP on a reporter plasmid assay using *skil*SBEs-Luc and UBmSNON. We observed that UBmSNON was recruited to the *SKIL* gene reporter similarly to WT HA-SNON, and it is very interesting that TGF- β was able to remove the UBmSNON protein from the *SKIL* promoter even though TGF- β did not cause its degradation (Fig. 4F). Thus, we demonstrated that *SNON* protein degradation is not a prerequisite for *SNON* to be removed from its target gene promoters. However, we also showed that it is important for TGF- β signaling that activated SMAD complexes remove *SNON* from the promoter to bind the promoter and fully

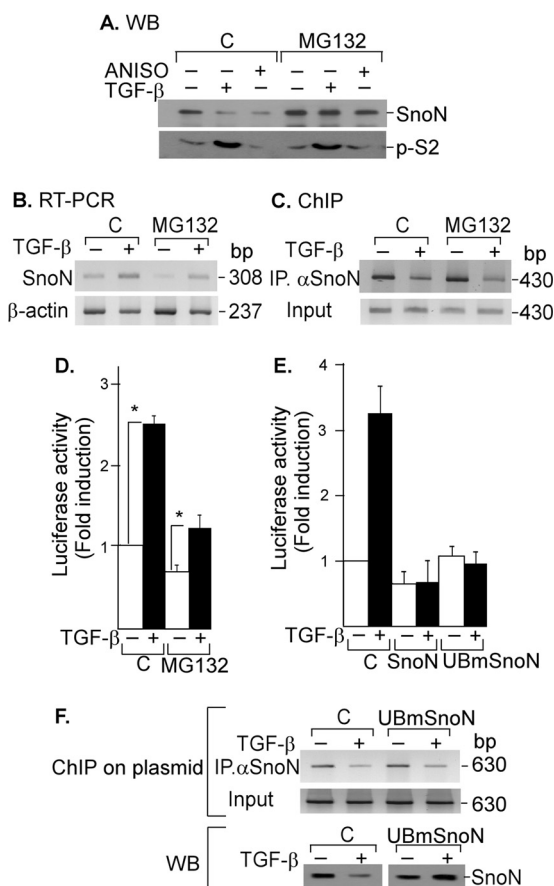


FIGURE 4. TGF- β signal removes SNON from SKIL gene promoter independently of its degradation. A, A549 cells were pretreated for 2 h without or with 50 μ M MG132 and then incubated for 45 min in the absence or presence of 300 pM TGF- β or 10 μ M ANISO. Proteins were immunoprecipitated with anti-SNON or anti-SMAD2 antibody followed by WB ($n = 2$) (A), or total RNA was isolated, and SNON (308-bp) and β -actin (237-bp) mRNAs were amplified by RT-PCR with specific primers ($n = 2$) (B), or ChIP assays were carried out using anti-SNON antibody, and PCRs were done with primers spanning SKIL SBE region (430 bp) ($n = 3$) (C). D, A549 cells transfected with the skilSBEs(408)-Luc reporter were pretreated for 2 h without or with 50 μ M MG132 and then incubated for 12 h with or without 100 pM TGF- β . Luciferase activity was evaluated and normalized using β -gal expression and is reported as -fold induction over control. Values are mean \pm S.E. (error bars) of three separate experiments in triplicate. *, $p < 0.05$ compared with control (C). E, to further analyze whether SNON degradation was required to regulate SKIL gene, we used UBmSNON, which is unable to be ubiquitinated or degraded. AD293 cells were transiently transfected with skilSBEs(408)-Luc reporter along with WT SNON or UBmSNON, and 24 h post-transfection, cells were incubated for 12 h with or without 100 pM TGF- β . Luciferase activity was evaluated and normalized using β -gal expression and is reported as -fold induction over control. Values are mean \pm S.E. (error bars) of three separate experiments in triplicate. F, AD293 cells were transiently transfected with the skilSBEs(408)-Luc reporter with or without UBmSNON. Cells were incubated for 45 min with or without 500 pM TGF- β , and a ChIP on plasmid assay was carried out using anti-SNON antibody for IP. PCRs were done with primers spanning the SBE region cloned into pGL3 vector (630 bp) (upper panel). Endogenous SNON and UBmSNON protein levels were detected by Western blot (lower panel).

induce SKIL gene expression as it has been suggested previously (17).

SNON Protein Negatively Self-regulates Its Expression at Transcriptional Level—To demonstrate that SNON can regulate its own expression at the transcriptional level, we used two different strategies to down-regulate SNON protein levels and evaluated their effect on SKIL gene expression. First, A549 cells were pretreated with or without 10 μ M ANISO for 1 h and then

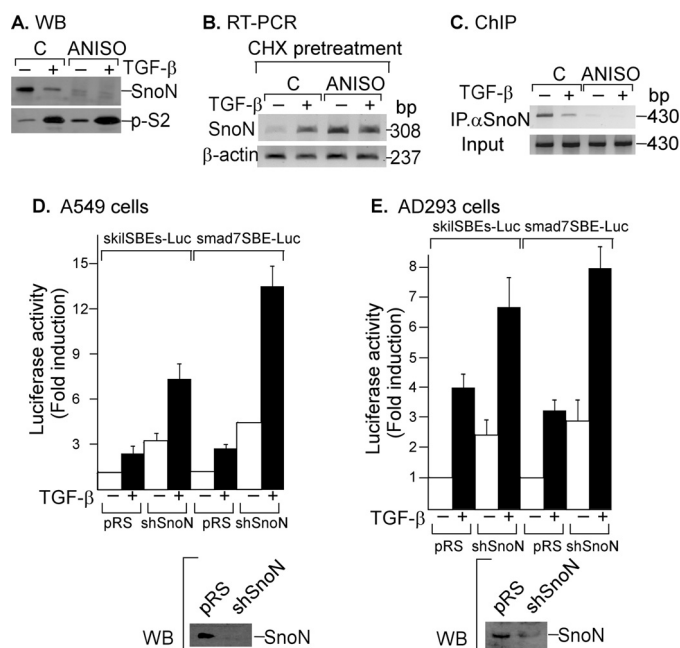


FIGURE 5. SNON negatively self-regulates its expression at the transcriptional level. A, A549 cells were pretreated for 1 h in the absence or presence of 10 μ M ANISO and then incubated for 45 min with or without 300 pM TGF- β . Whole cell protein extracts were immunoprecipitated with specific anti-SNON and anti-S2/S3 antibodies and then analyzed by immunoblotting with specific anti-SNON and anti-phospho-SMAD2 (p-S2) antibodies. B, A549 cells pretreated with cycloheximide (CHX) for 20 min were then pretreated for 1 h in the absence or presence of 10 μ M ANISO and then incubated for 2 h with or without 300 pM TGF- β ($n = 2$). Total RNA was isolated, and RT-PCR was performed with specific primers for SNON and β -actin. C, A549 cells were pretreated for 1 h without or with 10 μ M ANISO and then treated for 45 min with or without 500 pM TGF- β , and a ChIP assay was carried out using anti-SNON antibody for IP ($n = 3$). PCRs were done with primers spanning the SKIL SBE region. A549 (D) or AD293 (E) cells stably expressing an shRNA to knock down SNON were co-transfected with the skilSBEs(408)-Luc or smad7SBE-Luc reporter plasmid, and 24 h post-transfection, cells were incubated for 12 h in the absence or presence of 100 pM TGF- β . Luciferase activity was normalized using β -gal expression and is reported as -fold induction over control (C). Values are mean \pm S.E. (error bars) of three separate experiments in triplicate (upper panels). Endogenous SNON protein levels were detected in each cell type by Western blot (lower panels).

incubated for 45 min in the absence or presence of 300 pM TGF- β , and SNON protein levels were evaluated by immunoprecipitation (IP)/Western blot (WB) (Fig. 5A). These data showed that SNON protein was degraded after TGF- β or ANISO treatment as we reported previously (13, 16). Thus, we evaluated how SNON protein down-regulation induced by ANISO contributes to the regulation of SKIL gene expression. First, SNON mRNA levels were analyzed by RT-PCR in A549 cells pretreated for 20 min with 20 μ g/ml cycloheximide to inhibit protein synthesis and then incubated for 2 h in the absence or presence of TGF- β or ANISO. β -Actin mRNA (237 bp) was amplified as an RNA loading control. These data showed that TGF- β induced an increase of SNON mRNA levels, whereas ANISO increased basal and TGF- β -induced SNON mRNA levels (Fig. 5B). It is possible that SNON protein down-regulation induced by ANISO caused a derepression of SKIL gene. This was supported by the observation that endogenous SNON was not positioned on the SKIL promoter when A549 cells were pretreated with ANISO (Fig. 5C). These data suggest that SNON protein down-regulation by ANISO may relieve the

SNON-S4 Complex Represses Human SKIL Gene Expression

basal repression of *SKIL* gene and may facilitate the TGF- β effect on *SKIL* gene induction.

To validate the hypothesis that SNON was involved in basal repression of *SKIL* gene promoter, the endogenous SNON protein expression was knocked down using shRNA in AD293 and A549 cells. SNON knockdown cells (shSnoN) with low SNON protein levels (Fig. 5, D and E, lower panels) showed higher skilSBEs(408)-Luc reporter basal expression as well as higher TGF- β -induced activity (Fig. 5, D and E, upper panels). Because the levels of SNON expression could affect the expression of several TGF- β and SNON target genes, we also investigated the *SMAD7* gene expression. We observed that basal and TGF- β -induced activity of smad7SBE-Luc was also increased in SNON knockdown cells (Fig. 5, D and E, upper panels). Therefore, it is very likely that the levels of SNON protein may affect the basal expression of most of its target genes.

SMAD4 Protein Is Required to Induce SKIL Gene Expression by TGF- β and to Bind and Repress SKIL Gene Promoter by SNON—We evaluated the role of S4 in recruiting R-SMAD and SNON proteins to the *SKIL* promoter. SNON and SKI do not possess DNA binding ability, and they seem to be recruited to TGF- β -responsive gene promoters through their interaction with SMAD proteins, mainly S4 (41, 42). Moreover, the *SKIL* gene seems to be induced by TGF- β in either an S4-dependent or S4-independent manner (43, 44).

To study the participation of S4 in the regulation of the human *SKIL* promoter by TGF- β /SMADs and SNON, we used the AD293 cell line stably expressing pRetroSuper/shS4J hygro (shS4; cells with low levels of SMAD4 protein by RNAi) or pRetroSuper/shS4J hygro plus pBABE/Smad4J Rescue (S4R; cells that overexpress SMAD4 protein because *S4* mRNA cannot be degraded) (Fig. 6A, upper panel). TGF- β transiently increased SNON mRNA levels in AD293 cells, and the highest levels were observed between 2 and 4 h after treatment (Fig. 6A, middle panel). We also observed that TGF- β increased SNON mRNA levels at 2 h in control and S4R cells but not in shS4 cells where the induction of *SKIL* gene by TGF- β was reduced (Fig. 6A, lower panel). Interestingly, the basal expression of SNON mRNA was increased in shS4 cells with respect to control and S4R cells (Fig. 6A, lower panel). Our data indicate that S4 is necessary not only for repressing *SKIL* gene expression at the basal level but also for inducing its expression by TGF- β in these cells.

SNON protein levels were also evaluated in control and shS4 AD293 cells. We observed that its levels decreased at 45 min and then increased after 2 h of TGF- β treatment in control cells (Fig. 6B), whereas TGF- β was unable to induce SNON expression at 2 h in the shS4 cells (Fig. 6B). Phospho-SMAD2 levels were increased after TGF- β treatment in both control and shS4 AD293 cells (Fig. 6B). Furthermore, we also observed that *SKIL* gene promoter activity induced by TGF- β was dependent on S4 expression because this effect only occurred in control and S4R cells (Fig. 6C). TGF- β was also able to activate 3TP-Lux in a SMAD4-dependent manner (data not shown) as reported previously (43, 44). Intriguingly, SNON could not repress its expression when SMAD4 was absent, and as a consequence, the levels of SNON mRNA as well as the activity of *SKIL* promoter were increased (Fig. 6, A and C). Our results show that S4 pro-

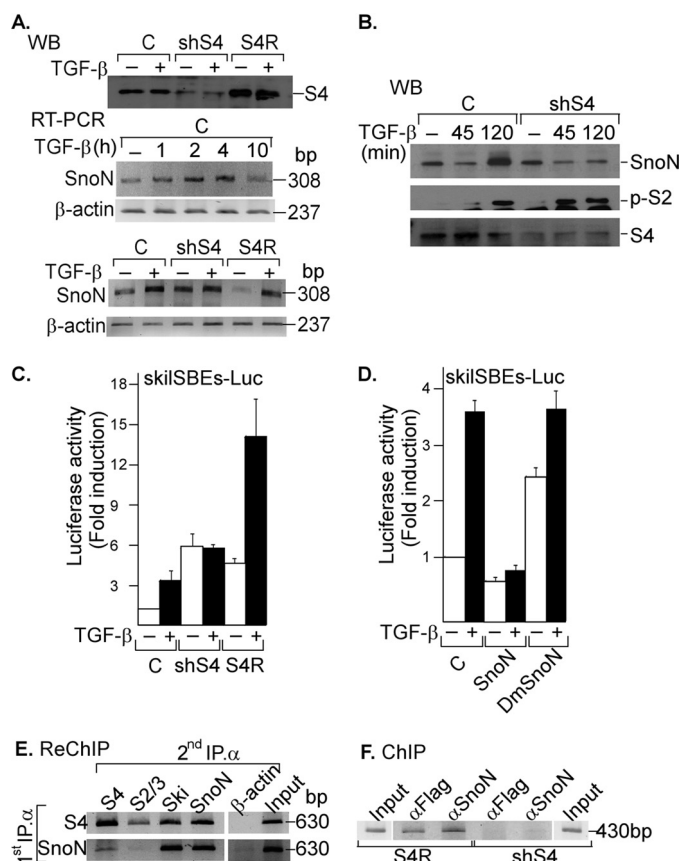


FIGURE 6. SNON depends on SMAD4 protein for *SKIL* promoter binding and repression. A, control AD293 cells (C) or cells stably expressing shSmad4 (shS4) or shSmad4 plus rescue S4 cDNA (S4R) were incubated for 2 h with or without 300 pM TGF- β . Whole cell protein extracts were subjected to IP with anti-S4 specific antibody followed by immunoblotting (upper panel). Total RNA was also isolated from control AD293 cells (upper and lower panels) or from shS4 or S4R AD293 cells (lower panel) incubated with or without 300 pM TGF- β for different times, and SNON (308-bp) and β -actin (237-bp) mRNAs were amplified by RT-PCR with specific primers ($n = 2$). B, control AD293 cells or stably expressing shSmad4 (shS4) AD293 cells were incubated for different times with 300 pM TGF- β . Whole cell protein extracts were subjected to IP with anti-SNON, anti-S2/S3, or anti-S4 antibody followed by WB with anti-SNON, anti-phospho-S2 (p-S2), or anti-S4 antibody ($n = 2$). Control or shS4 AD293 cells were transiently transfected with skilSBEs(408)-Luc reporter plasmid with or without plasmids bearing cDNA for SMAD4 rescue (S4R) (C), and AD293 cells were transiently transfected with skilSBEs(408)-Luc reporter along with plasmids bearing cDNA for WT SNON or DmSNON (D). Twenty-four hours post-transfection, cells were incubated for 12 h in the absence or presence of 100 pM TGF- β . Luciferase activity was normalized using β -gal expression and is reported as -fold induction over control. Values are mean \pm S.E. (error bars) ($n = 6$). AD293 cells were transiently transfected with skilSBEs-Luc reporter, and 48 h post-transfection, a re-ChIP assay was carried out ($n = 2$) (E), or a ChIP assay was carried out in shS4 and S4R AD293 cells (F); both assays were performed using anti-S4, anti-FLAG, or anti-SNON antibody for the first IP; anti-S4, anti-S2/S3, anti-SNON, or anti-SKI antibody for the second IP; and anti- β -actin antibody as a control. PCRs were performed with primers for pGL3 plasmid bearing *SKIL* promoter (630 bp) or for *SKIL* promoter (430 bp).

tein is needed to induce *SKIL* gene expression by TGF- β and for the self-repression caused by SNON in AD293 cells.

To further evaluate the relevance of SNON-SMAD interaction in the regulation of *SKIL* gene expression, we constructed a double mutant of SNON (DmSNON) unable to associate with R-SMAD and SMAD4 (41). We observed that, in contrast to WT SNON, the DmSNON was unable to repress both basal and TGF- β -induced activity of skilSBEs(408)-Luc reporter (Fig. 6D). Our data indicated that SNON requires interaction with

SMADs to repress the expression of *SKIL* gene, and previous studies have shown that SKI requires S4 to inhibit the *SMAD7* gene promoter (12, 42). To evaluate whether the regulation of *SKIL* gene by SNON also requires its association with SMAD4, a re-ChIP assay on reporter plasmid was carried out in AD293 cells expressing skilSBEs-Luc using anti-SNON and anti-S4 antibodies for first and second IPs. Our results showed that endogenous SNON and SKI associate with endogenous S4, and this association appears to mediate the binding of SNON and SKI proteins to the *SKIL* promoter (Fig. 6E). We also observed by endogenous ChIP assays in S4R and shS4 AD293 cells that SNON can bind the *SKIL* promoter only when S4 is expressed (Fig. 6F). It is possible that SKI and SNON may also depend on S4 to bind to other TGF- β target gene promoters as occurs with the *SKIL* gene. We also found that SNON interacts with S4 to negatively regulate its own basal and TGF- β -induced expression.

The Absence of SNON-SMAD4 Complex Affects the Biological Outcome of TGF- β Signaling—In the epithelial AD293 cell line, we observed that TGF- β requires S4 to induce *SKIL* gene expression, whereas SNON depends on S4 protein for binding and repression of *SKIL* gene promoter. Because the response of cells to TGF- β depends on the cell context, we evaluated the effect of TGF- β on *SKIL* gene expression in two different cell types: A549 cells that undergo epithelial-mesenchymal transition in response to TGF- β and exhibit a high level of SNON protein and a low level of SKI protein and the colon cancer cell line SW480 that lacks S4, has a mesenchymal phenotype, and contains very high levels of both SNON and SKI proteins that are not down-regulated in response to TGF- β . We first examined the SNON expression levels in these cells by WB. The levels of SNON protein were very high in SW480 cells compared with AD293 and A549 cells (Fig. 7A). Thus, we decided to study the role played by SNON and S4 expression levels in the formation of SNON-S4 complex as well as the role of this complex in the regulation of human *SKIL* gene by TGF- β in different cellular contexts.

We evaluated SNON protein levels after TGF- β treatment for different times in shS4 and S4R A549 cells (Fig. 7B), SW480 cells lacking endogenous S4 expression, and SW480 cells transiently expressing S4 (Fig. 7C). We observed that SNON protein levels decreased at 45 min and then increased at 2 h in both shS4 and S4R A549 cells; however, we also observed a higher basal level of SNON protein expression as well as high levels of SNON protein after 4 h or longer times of TGF- β treatment in shS4 cells than in S4R cells (Fig. 7B).

In the case of the SW480 cell line, both control or overexpressing S4, SNON protein levels were just slightly increased in response to TGF- β even though the phospho-S2 levels were highly increased (Fig. 7C). Interestingly, TGF- β was unable to cause SNON degradation at any time point even though the phospho-S2 levels remained elevated until 20 h after TGF- β treatment (Fig. 7C, upper panel). Also, we observed a strong interaction among SNON, SKI, and phospho-S2 in control SW480 cells after TGF- β treatment (Fig. 7C, lower panel). However, we observed an interaction between SNON and S4 only in SW480 cells transiently expressing S4. Furthermore, TSA was unable to increase SNON mRNA levels in SW480 cells

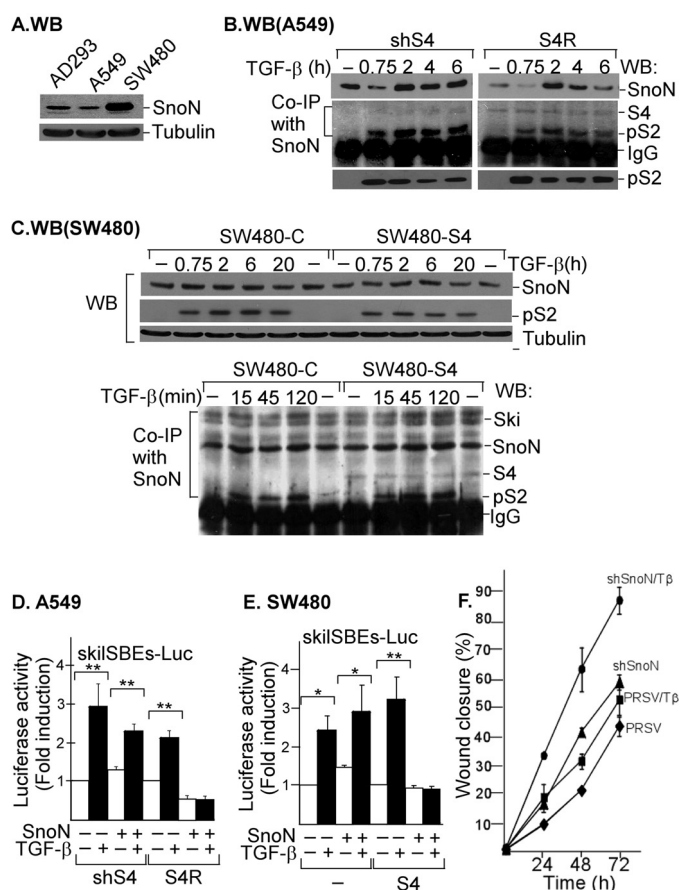


FIGURE 7. The absence of SNON-SMAD4 complex affects TGF- β signaling outcome. A, the levels of SNON protein were detected by WB in total protein extracts from AD293, A549, and SW480 cells. The levels of tubulin were used as a loading control ($n = 3$). For IP/WB assays, the following cell types were incubated for different times with or without 300 pM TGF- β : A549 cells stably expressing shS4 or expressing shS4 plus rescue S4 cDNA (S4R) ($n = 2$) (B) and total cell lysates from control or transiently expressing S4 SW480 cells were subjected to WB (upper panel) or IP with anti-SNON or anti-SMAD2/3 specific antibody (lower panel) followed by WB with anti-SNON, anti-SKI, anti-S4, or anti-phospho-S2 (pS2) antibody ($n = 2$) (C). For reporter gene assays, the following cell types were transiently transfected with skilSBEs(408)-E1B-Luc: A549 cells stably expressing shS4 or expressing shS4 plus rescue S4 cDNA (S4R) (D) and control (–) or transiently expressing S4 or SNON SW480 cells (E). Transfected cells were then incubated for 12 h with or without 100 pM TGF- β , and then luciferase activity was measured and normalized using β -gal expression. Data are reported as -fold induction over control. Values are mean \pm S.E. (error bars) of three separate experiments in triplicate (upper panels). *, $p < 0.05$; **, $p < 0.01$ compared with control (C). F, confluent and serum-starved A549 cells stably expressing empty vector pRSV (◆ and ■) or shS4 (▲ and ●) were wounded, and then the wound closure was monitored for different times in the absence or presence of 50 pM TGF- β (T β) (■ and ●). The graph shows a representative experiment, and data are shown as percentage of wound closure (mean \pm S.E. (error bars), $n = 3$).

(data not shown), supporting the idea that in these cells the basal SNON-S4 repressor complex is absent.

Interestingly, we observed that the induction of *SKIL* gene reporter by TGF- β was independent of S4 expression in A549 cells because this effect occurred in both shS4 and S4R A549 cells (Fig. 7D). Similar data were obtained in SW480 cells (Fig. 7E). Additionally, the analysis of smad7-Luc reporter showed a similar regulation (data not shown). Intriguingly, when SMAD4 was absent or its levels were low, SNON could not repress its own expression, and as a consequence, the activity of *SKIL* promoter was increased (Fig. 7, D and E). Our results showed that

S4 protein is always indispensable for the *SKIL* gene repression caused by SNON.

To further study the relevance of SNON levels in regulating the response of the cell to TGF- β , we evaluated the migration of A549 cells by using a wound healing assay. Confluent and serum-starved A549 cells stably expressing empty vector (pRSV) or shSnoN were wounded, and the wound closure was monitored for different times in the presence or absence of TGF- β (Fig. 7F). We observed that the knockdown of SNON increased the migratory phenotype of A549 cells and promoted the TGF- β effect. These data show the relevance of SNON levels to control TGF- β signals.

DISCUSSION

SNON is a negative regulator of SMAD transcriptional factors that may control many actions of TGF- β (1–9, 41, 42). Changes in SNON levels have been associated with diverse physiological processes such as embryonic development, hepatic regeneration, and muscular differentiation as well as with some diseases such as fibrosis and cancer, which are also related to altered TGF- β signaling (26–30, 45). Much evidence suggests that the regulation of SNON expression is essential in homeostasis and very complex. So far, TGF- β and hepatocyte growth factor are the main known signals that can induce SNON expression (25, 46). Because SNON expression might be critical in diverse physiological and pathological processes, here we aimed to describe the molecular mechanisms controlling human *SKIL* gene expression by the SNON-SMAD4 complex and TGF- β signaling.

Our bioinformatics analysis showed that the TRE spans the *SKIL* gene promoter. Also, the TSS of *SKIL* gene was located between SBE3 and SBE4 of TRE by partial cloning of the 5'-UTR of SNON mRNA by RT-PCR assay. A previous report showed by ChIP assay that TGF- β induced RNA polymerase II recruitment to the TRE of *SKIL* gene (47), which supports our finding that the TRE region spans the core promoter of *SKIL* gene. Intriguingly, TGF- β -responsive *SKIL* gene promoter is spanned by four groups of SBEs (supplemental Fig. S1). The affinity of SMAD proteins for one SBE is very low, but the presence of multiple SBEs probably increases the SMAD binding affinity to improve transcriptional activation through cooperative interactions between multiple SMAD-SBE contacts by the activated SMAD complex as suggested previously (1, 2, 4, 11–13, 48, 49).

Our analysis also showed that the ATG is located in the 5'-half of the second exon of the *SKIL* gene, whereas the first exon is small, GC-rich, and non-coding, and the first intron is very large. Interestingly, these particular characteristics of *SKIL* gene show that the first exon and first intron contain the previously identified regulatory sequences, the SMAD inhibitory element and hepatocyte growth factor-responsive element, which were identified as relevant elements for the regulation of *SKIL* gene expression by TGF- β and hepatocyte growth factor, respectively (25, 46, 50). We also found that the *SKIL* promoter is a TATA-less core promoter, and it is spanned by a large CpG island (supplemental Fig. S1) (51).

In the TGF- β pathway, SKI and SNON are two important SMAD corepressors (5, 6). SNON can form homodimers or

heterodimers with SKI, and they play a crucial role in cellular transformation and transcriptional repression (7, 8). Interestingly, SNON and SKI protein levels can be regulated by TGF- β because the UPS rapidly degrades them upon ligand stimulus (15–22). In addition, TGF- β induces an increase of SNON mRNA and protein levels at treatments longer than 1 h (13, 23). Our work focused on demonstrating that SNON protein negatively regulates the basal expression of *SKIL* gene and its induction by the TGF- β /SMAD pathway; we also demonstrated that this last event is part of a negative feedback loop generated by TGF- β itself (Figs. 3 and 5). Our data support the idea that the transcriptional regulation of *SKIL* gene by SKI and SNON could be considered as a general mechanism to control other TGF- β target genes because the *SMAD7* gene appears to be similarly regulated by SKI and SNON (Fig. 3) (12, 13). Therefore, it is clear that the transcriptional self-regulation of SNON could potentially affect the expression of many of its target genes, which can be analyzed when more SNON target genes are identified.

Because SNON and SKI cannot bind directly to DNA, we explored how these corepressors bind to the *SKIL* promoter. It has been reported that SKI binds DNA through S4 to repress the basal activity of *SMAD7* gene independently of R-SMADs (12, 13, 52). Here, we demonstrated that SNON negatively self-regulates its expression because SNON binds to the *SKIL* promoter and recruits a repressor complex that also contains SKI, SMAD4, and proteins with HDAC activity. We also provide evidence that activated R-SMADs promote SNON protein removal from *SKIL* promoter independently of inducing its degradation. However, SNON degradation via the UPS is important to decrease the availability of SNON protein that may compete with activated R-SMAD complexes for TGF- β target gene promoters.

SMAD4 is not always required in SMAD transcriptional complexes to activate TGF- β target genes, and some TGF- β target genes can be differentially regulated by S4 and R-SMADs (43, 44, 53, 54). In fact, tumor cells deficient in S4 or expressing mutant S4 or cells with low levels of S4 due to shRNAs display a differential regulation of some but not all TGF- β target genes (43, 55, 56). Interestingly, *SKIL* was identified previously as a TGF- β target gene that may be regulated via an S4-dependent or S4-independent manner (43, 57). Here, we showed that the induction of *SKIL* gene by TGF- β effectively may rely on S4, but it depends on the cell type, which is similar to previous reports (43, 57).

Notably, the self-repression mediated by SNON is clearly S4-dependent because SNON was unable to inhibit the basal and TGF- β -induced *SKIL* gene expression when S4 protein levels were decreased in all cell types tested. After restoring S4 expression or when S4 was overexpressed, it was possible to rescue the interaction of SNON with S4 and the recruitment of S4-SNON complex to *SKIL* promoter and as a result to decrease SNON mRNA levels. We confirmed that SNON associates with activated R-SMADs, S4, and SKI. Thus, when SNON does not bind S4, then it cannot repress the *SKIL* promoter. Using a re-ChIP assay, we confirmed that SNON and SKI proteins in combination with S4 bind and repress the *SKIL* promoter basal expression. Furthermore, we demonstrated that the absence of

the SNON-S4 repressor complex affects the regulation of *SKIL* gene expression by TGF- β . Because S4 is a functional partner for SNON and SKI, it is very likely that S4 is also required for transcriptional repression of many other TGF- β target genes.

It is possible that the self-regulation of SNON expression could be affected in some cellular contexts. It was reported previously that a prolonged induction of SNON expression by TGF- β in fibroblast plays a critical role for cell oncogenic transformation; intriguingly, in these cells, the negative feedback loop seems to be absent (25). Nevertheless, we have demonstrated that when high levels of *SNON* mRNA are observed in cells where SMAD proteins remain activated for a long time, it is possible that the negative feedback loop is functioning to stop the gene transcription, but at the same time, a mechanism controlling the stability of mRNAs might be also participating.

The absence of this self-regulation of SNON could be a new mechanism causing up-regulation of TGF- β inhibitors such as *SMAD7* and *SKIL* (*Sno*) genes in some diseases such as cancer, and it may favor TGF- β resistance in cancer cells by affecting the regulation of TGF- β target genes. This new mechanism could also be evident mainly in cancer cells lacking SMAD4 such as those from colon and pancreas (58) where the absence of the SNON-SMAD4 repressor complex might be responsible for the changes observed in cell phenotype (25, 26). Furthermore, it is highly probable that in those cancerous cells lacking SMAD4 the functions of both corepressors SNON and SKI are impaired.

In summary, SNON and SKI are bound to the *SKIL* gene promoter to repress its basal expression in a manner similar to that for *SMAD7* gene regulation. The *SKIL* gene is a target of SKI and SNON corepressors, and both seem to be involved in maintaining the *SKIL* promoter in a repressed state in the absence of TGF- β signaling. That this effect depends on the association with SMAD4 is noteworthy. In contrast, upon TGF- β stimulus, SNON and SKI are removed from the *SKIL* promoter and replaced by the activated SMAD complex. Thus, TGF- β positively regulates the human *SKIL* gene expression through the S2-S4 complex. Later, after longer TGF- β treatment, SNON protein levels are increased, and SNON binds back to the *SKIL* promoter. Thus, SNON functions as a negative feedback control regulating *SKIL* (*Sno*) expression (supplemental Fig. S5). This regulation of SNON levels could be critical for appropriate control of the TGF- β signal and for cell homeostasis. Thus, any deregulation of this negative feedback loop might be involved in the development of diverse diseases such as fibrosis and cancer.

Acknowledgments—We thank Drs. Claudia González-Espinosa, Marco A. Briones-Orta, and Jacqueline Hernández-Damián for helpful discussions. We appreciate the kind gifts of plasmids from Drs. J. L. Wrana (Mount Sinai Hospital, Toronto, Ontario, Canada), H. F. Lodish (Whitehead Institute, Cambridge, MA), and M. Klüppel (Northwestern University, Chicago, IL). We thank Drs. Rosa Navarro and Jorge Ramírez-Salcedo (Instituto de Fisiología Celular, Universidad Nacional Autónoma de México), and José Vázquez-Prado (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional) for reagents. We also thank Dr. L. Ongay and members of Unidad de Biología Molecular (Instituto de Fisiología Celular, Universidad Nacional Autónoma de México).

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SUPPLEMENTARY DATA

TRANSFORMING GROWTH FACTOR- β /SMAD TARGET GENE SKIL IS NEGATIVELY REGULATED BY THE TRANSCRIPTIONAL COFACTOR COMPLEX SNON/SMAD4

Angeles C. Tecalco-Cruz, Marcela Sosa-Garrocho, Genaro Vázquez-Victorio, Layla Ortiz-García, Elisa Domínguez-Hüttinger and Marina Macías-Silva

Fig. S1. *The predicted human skil gene structure assembled on the basis of bioinformatic and experimental analysis.* (A) The localization of the promoter of *skil* gene was predicted by using a BLAST alignment between the clone of human chromosome 3 (Accession No. AC073288) and the clone of human SnoN mRNA (Accession No. NM_005414.4), and also by the analysis of the sequences by using several TSS-prediction programs. All the results indicated that the promoter of *skil* gene is located in a region ~2.4 kb upstream from the translation start site ATG (Start). The promoter region includes the TGF- β -responsive element (TRE) that contains several Smad-binding elements (SBEs). The *skil* gene includes 7 exons and 6 introns. The SnoN pre-mRNA sequence (Accession No. NG_03037.1) shows that the non-coding Exon1 and part of Exon2 encode the 5' UTR, whereas ATG is localized at 5' half of Exon2. (B) Human SnoN mRNA transcript structure was assembled on the basis of GenBank sequence (NM_005414.4), and this sequence exhibits two putative poly-A sites at the 3'UTR (~4.4 kb). (C) Localization of some regulatory sequences present upstream of the *skil* gene translation start site ATG (+1) are indicated such as the TRE (SBE 1–4), SIE and HGF-responsive region. The location of a large CpG island spanning the TSS and promoter region is also indicated (----).

Fig. S2. *Localization of TSS at the TRE region of skil gene promoter and partial cloning of 5' UTR of SnoN mRNA.* (A) The positions of primers used are indicated by arrows on *skil* gene map (forward primers: A, B, C and D, and reverse primers E and F). ATG (+1) is localized at 5' half of Exon2, whereas the TSS is located at the region between the SBE3 and SBE4 of TRE. (B) Total RNA from HepG2 cells treated 1 h with or without 300 pM TGF- β was used as template for RT-PCR assays. Total RNA was pretreated (+RT) or untreated (-RT) with Reverse Transcriptase prior to obtain the cDNA, and then the fragments of 5'UTR were amplified by PCR using the reverse primer E in combination with the forward primers: A, B, C or D (sequences are shown in Table S2). (C) cDNA obtained from HepG2 cells was used as template to amplify 5'UTR of SnoN mRNA (839 bp) by PCR using the reverse primer F and the forward primer B; human genomic DNA was also used as template for PCR assay (2776 bp). (D) A fragment (~803 bp) of the 5'UTR of SnoN mRNA was cloned and sequence verified. Non-coding Exon1 and 5' half part of Exon2 form the 5' UTR of SnoN mRNA.

Fig. S3. *Blast alignment of human skil and mouse sno gene partial sequences.* The Blast alignment of human *skil* and mouse *sno* gene partial sequences shows that both sequences are highly conserved (* indicates identity). The promoter region was identified and it is spanned by a TRE region (–3041/–2716), which contains several Smad-binding elements (SBEs). The gene regulatory elements are underlined: the translation start codon (ATG) is located in the second exon, the first exon is non-coding, the first intron is large (~1933 bp) and contains conserved regulatory elements such as SIE (Smad-inhibitory element) and HGFR (HGF-responsive element).

Fig. S4. *Putative transcription factor binding sites identified on the human skil gene promoter by bioinformatic.* Several putative binding sites for a variety of transcription factors were identified on the TRE of *skil* promoter using ALGGEN program. Many of these transcription factors are known Smad partners and could be involved in the regulation of SnoN expression by other signaling

pathways. The percentages indicate the degree of conservation between the putative transcription factor binding sites between *Homo sapiens* and *Mus musculus* sequences as determined with NCBI-BLAST.

Fig. S5. Proposed model of the molecular mechanism involved in the regulation of SnoN expression. SnoN and Ski together with Smad4 bind to *skil* gene promoter to repress its basal expression by recruiting HDACs. Upon TGF- β signaling, SnoN and Ski are removed from *skil* gene promoter and replaced by activated Smad complexes, they are also targeted for degradation via UPS. After longer TGF- β treatment (~2 h), SnoN mRNA and protein levels are increased, and newly synthesized SnoN protein binds back to the *skil* gene promoter to repress its own expression as part of a negative feedback loop generated by TGF- β itself.

**TRANSFORMING GROWTH FACTOR- β /SMAD TARGET GENE *SKIL* IS NEGATIVELY REGULATED
BY THE TRANSCRIPTIONAL COFACTOR COMPLEX SNON/SMAD4**

Figure S1

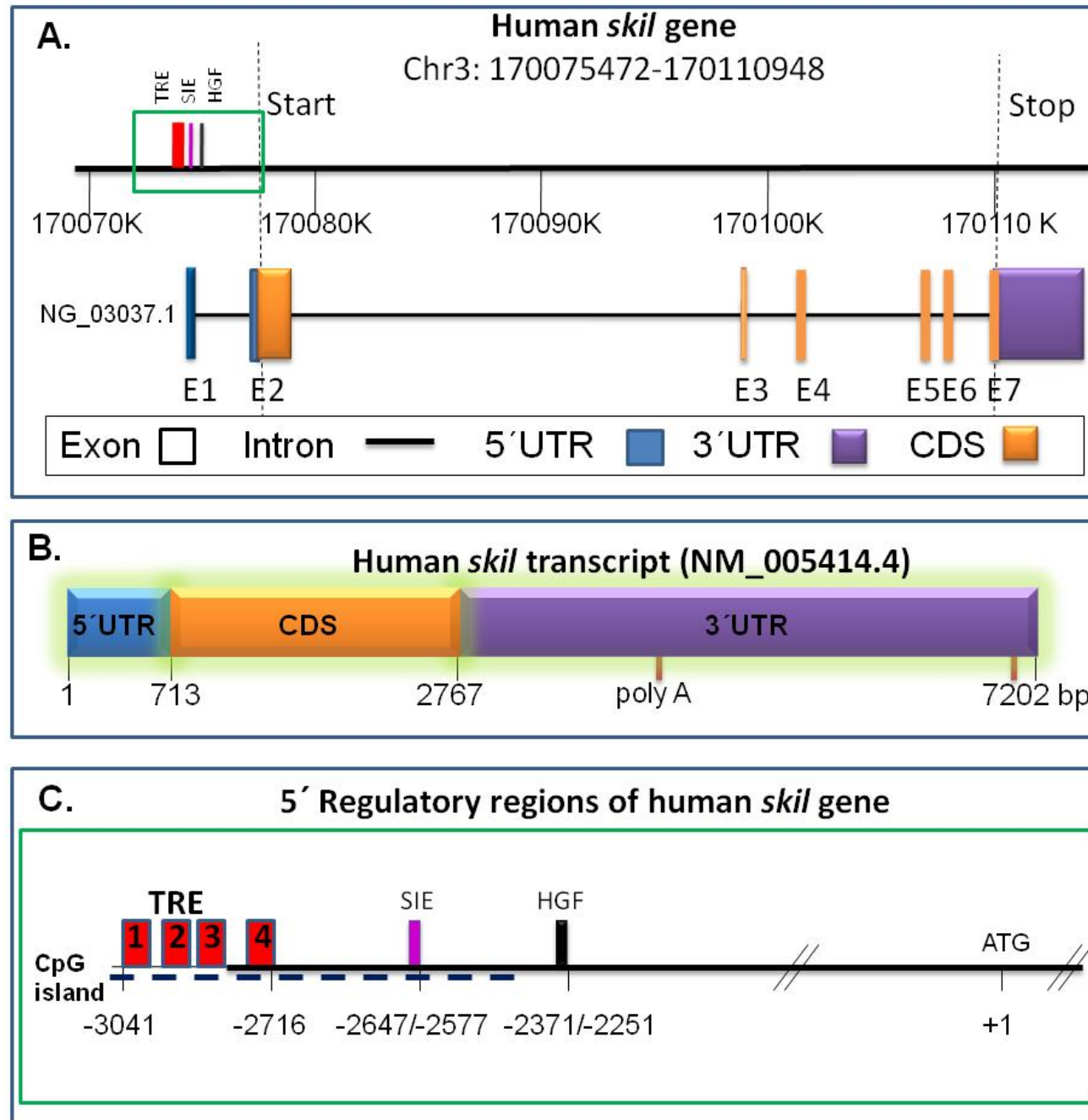


Figure S2

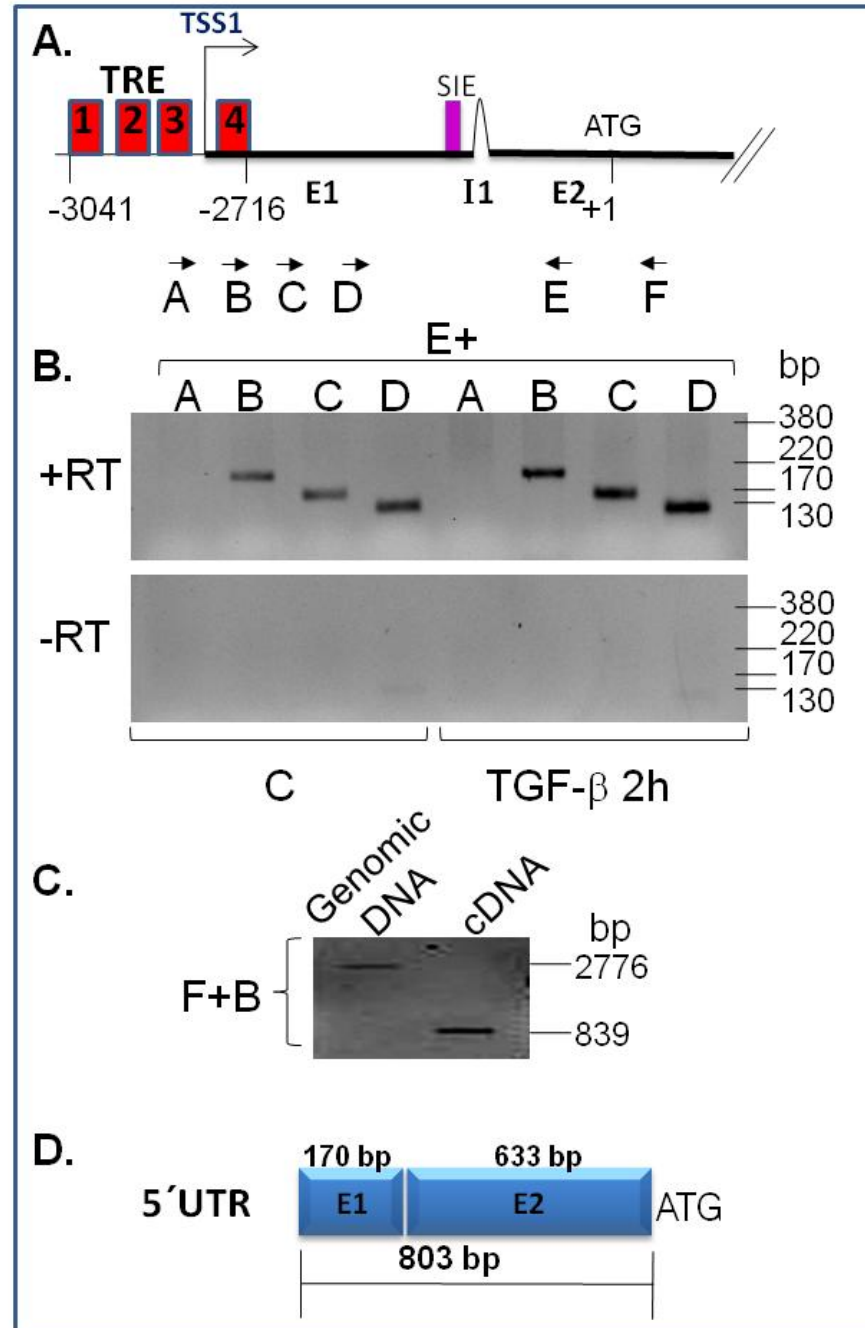


Figure S3

Mouse	GACCTCGGGCGCGGCCAGCTGCCGGGGTCGCGCACAGTGCAGCCCGCCGC-GCCCTGCCC	-2827
Human	TGCCTCCG-CCCTGCCGGCTGCTGGGGTCGCGCACAGTGCACCGCGGGCTGCCCTGCCC	-3002
	**** * * * **** *****	
Mouse	CCTCTCCCCCTTCTCACCCCCCGCCTCGCAGCGCGGGCGGCCGGCGGCCAGGGCGCCC	-2767
Human	CCTCTTCCCC-----GCTGCGCGGGCTCCGAGCGGCCAGGGCGCCC	-3171
	***** **	
Mouse	CCAGGTCTGGCCCGTCGCCACACGGCGGGCGGTGGCGAGGGCGG-----CCCCAG	-2715
Human	CCGCGTCTGGCCCATCACCACAGAGCGGCGGGTGGGGCGGGCGGGCGGTGGCCCCAG	-3111
	** ***** ** ***** ***** * **** *****	
Mouse	ACAGCCGGCTGAGCGACTCGAGGAAGGAAGGAGGG-----CGGCGGGGTGGGGCCG-	-2663
Human	ACAGCCGGCTGGGCGACTAGAGGAAGGAAGGAGGGAGGGCGCGGTGAGGGTGGGGTGGG	-3051
	***** ***** ***** ***** *** * ***** *	
SBE1		
Mouse	-AGGGAACATCCTGTCTGAGCCGGG--CACCGCAGACAGCGCGTGCGCCAGCCGC	-2606
Human	GAGGGAACATCCTGTCTGCGCCGGGTGCACCGCAGACAGCGCGCGCGCCAGCTTC	-2991
	***** ***** ***** ***** *****	
SBE2		
Mouse	ACGGCGCGCCGGCCAGCAGCGCGGGGTACGCGCGAGGGGGCGGGGAGCGGGAGCACG	-2546
Human	ACGGCGCGCCGGCCAGAAGCGCCGGGCGCGCCTGGGGGAGGGG-----CA	-2942
	***** ***** ***** ** ***** ***** *	
Mouse	CGCGTGCAGCGCGGAGGCGCCAGGCGCGCGCCCGGTGCGGGCTGGGGACGCGCGCGCAG	-2486
Human	CGCGCGCGAGACGAGCGGGCCAGGGGGCGCTCTGA--GGAGCC--ACAATAGGCCAG	-2888
	**** * * * * * ***** * * * * * * * * * *	
SBE3		
Mouse	CCTGCAGTGTGCAGCCCGCGGGAGCCACAATAGGCCAGCGCGCGCGCGCCGAGGGG	-2426
Human	ACGGCA-CGCGCGTCCACGAGGGGGCCGGGAGCCCGAAGTGGCGGCGGCGCGACGACG	-2829
	* *	
Mouse	CTGGCTGGCGCGGGGCCGCG-CGGCGCCGCTGGCTTCGTTCCCTCCCTCCCTCCCTCC	-2369
Human	CCGGCGGGCCCGCGCGCGCGCGCGCCGCTGGCTCCGTTCCCTTCCCTTCCCTTCCCT	-2769
	* *	
SBE4		
Mouse	GCCGCCCTCGCTGTCCCCCGGCGGGCCGAGACGGCGGCGGTCTGCGGGAAGCCGTGT	-2309
Human	GCCGCCCTCGCTTCCCCCGGCGGGCCGAGACGGCGGCGGTCTGCGGGAAGCTGTGT	-2709
	***** ***** ***** ***** *****	
Mouse	--CTCCGAGTGACGTGGGCGGGCCGCGGGCCGGGTGACGTCAGAGG-CTGTGTGTAGCG	-2252
Human	GTCTCCGAGTGACGTGGGCGGGCCGAGGACTCGGTGACGTCAGAGGGCTGTGTGTAGCG	-2649
	***** ***** ***** ***** *****	
EXON1		
Mouse	ATGTGTGTGGGGTTCGGAGCGGCGCCGGCACAGC-GAAGGCGGCTGGCGAGCGACGGCGA	-2193
Human	ATGTGTGTGGGGTTCGGAGCCGCGCCGGCACAGCCGAAGGAGCGGGCGAGCGGCGACGG	-2589
	***** ***** ***** ***** *****	
SIE		
Mouse	CGGCGACGGCGGGCACAGGTGCGGCGCAGGTCGCGAGGG-GACGCGA--GGGGCGTGCG	-2136
Human	CGGCGCGGGCGGGCACAGGTGCGGCTCCGCTTACGGCGGCGACGCGGCGGAGGCGGCGG	-2529
	***** ***** ***** ***** *****	
INTRON 1		
Mouse	GCCTCTGGGGGCGCTGA-----GGCGGCGGCGCGGGGGTGATGGGGAGG	-2091
Human	GAGGCCAGAGCGCTACCTCCTAGGAGCCCGACGGCGGCGACAGGGGCGGTGGGGAAT	-2469
	* *	
Mouse	GCACAGAGCCCGG-GGGTCTGCCCGGGCGCGAGGGCCCTGAGTGGGGCGTCCGGGATGCG	-2032
Human	TCACAAAGCTCGTCGAGCTTACCCGGGCACCGAGG-CCCGAGTAGGGGGTTTGG-ATGCG	-2409
	**** * * * * * * * * * * * * * * * * * * *	

		Sp1A	
Mouse	TGGGGGTCCCGGGCGGGGTTCGCGGGCACAGGG--CGCGCGGGGGTCCCACGGGTGT	-1975	
Human	CGG--GTCCCGGTGGGGTTCGGGCCAGAGGGGTTCGCTAGGCGGGTGTTCACGGCTGA	-2351	
	** ***** ** ** ** *		
		CRE1	
Mouse	ACAGGCCTCGCGGATTCTGGGACGGGTTCAGTGGGAGGGGATGGCGGTGCACTTGACCCG	-1915	
Human	TCACGCTTCGTTGGCCCCAGGCGGTGGGCGCCGGCAGGGCAGGGCGCTGTAGTTGACGGG	-2291	
	** * * * *		
		CRE2 Sp1B	
Mouse	AGCCGCCGGGACCCAGGAGACCTGGACCCCTGGTCATCGCCGCAATCCCCCGG-CCGTCTG	-1855	
Human	AGCCGCCGGGACCCAGGAGACCCGGACGCCCGTCAACGCCCCTCCCCAGTCCAGCAG	-2231	
	***** ***** ***** ** *		
Mouse	CGCTCGGCGGGGATGCCCCGGGCTGGCCCCAGCTC-TAAGCGTCCGGCGACGCGGGCTTC	-1796	
Human	CGCTCCGCGGGGATGCCCTGGTCTCGACCCGGTCCGTCAGCCCCCGGCGGCAGGGATC	-2171	
	***** ***** ** * * * *		
Mouse	CGGGCTTGGCGCCTAACCTGCCCGCGCCCCCTGTGGGTGCCACGGCTCAGGGTGGGTCAAG	-1736	
Human	CGCGCCCCGGCGCCTAAACT-----GCCCTGGTGTCTCTTCGTTCTGGCCCCGGGCTGG	-2117	
	** * ***** ** * * * *		
Mouse	TGGGGGGAGGCGCAGCCCT-GGGGCGCCTAGGTAACCCTAGAGAACTTCCCCGGGGCCGG	-1677	
Human	CGCGGGGAGGTGCAGCATTAGGGGCGCTGAGGTGACACCCGAGAACTTCCAGGGAGAGG	-2057	
	* ***** * ***** ** *		
Mouse	GGGCGTGGGGT-----ATTTCCACCG-----GCGGCCCCCGCAGTGGGGCGATGTGG	-1630	
Human	GTGCGCGGGGGGGTGGATTTCCACAGTCTTCCCGCGGCCCCCGCGGGGGGGCGGTGTTG	-1997	
	* ** * * ***** *		
Mouse	GGAGGGCACTTAGGGTGATCCCAGTCACAGAGGTGGGGGTGGGAGGACTCTACTT---	-1573	
Human	GGAGGGCACTTGGGGTTATCCCAGGCTCGCCGGTGGGGGT-GGGGGGACTCTCCCTTTA	-1938	
	***** ***** * * ***** ** ***** *		
Mouse	---CCTCTGCCCCCTCTCAC-CTCACC---AGACGCCGGGAGTCCCCTGCTTCCCTGTC	-1521	
Human	CTTCCCTCCACCCCTTGACACCCACCCAGGCTTCCCGTGTCCCTTGTCTCCCTCT	-1878	
	*** ***** ** * * * * * * * *		
Mouse	CTTTGGGTTGATGATGAGTGGGTGTCTCTGCGTGTGCG-----CAGGGCTGTGGGGG	-1469	
Human	CTCCGGGTTAGAGATAGGTGGGTGGCTTTGAGTGGGCGGAGGACTTCAGGTTTATAGGGG	-1818	
	** ***** ** ***** ** * * * *		
Mouse	CGCCCCCTGGTCTAGGTCATGCCTTCTGCCCTTTTCTTGGCTCGCCTTGGCTGAGGTGT-	-1410	
Human	CTCCCCCATCTAGGTCATGCCTCTTGCTCTCTTCCCTTGGCCTGTGTTGAGTAAGGTGTG	-1758	
	* ***** ***** ** * * * * * *		
Mouse	-CCCTGTAGTGG-CGAAAGTCACAGTACGGTGGCTAGGTGGCTTTTCGTCCATAATTCTCT	-1352	
Human	TTCTGTGGTGGACGAAAGTCT--GTACGATGGTT-GGTGGCATTGGTCCTTAATCCTTT	-1701	
	***** ***** ***** ** * * * * * *		
Mouse	GGGTTTGTGTTAACATAAATTGCTGGGAGTTTAG-AAGCAAAGCTTTTTATTGTGCGCT	-1293	
Human	GGGTCTGTTTAAAGGTAAAGTGCTGAGAGTTTGGGAAGCAAAAATTCTATTATTGGAT	-1641	
	**** * * * * ***** * ***** ** ***** *		
Mouse	TT-TACTGGGTTTTCTCCAGGGAGGGAAGTGGATGAATACTCTTTT--TCGCCACTCTGC	-1236	
Human	TTCTTTTGGCTTTTCTCTCAAGAAGGAAGTGAAAAAGAGTCTTTTCTTTTCCCTCTTC	-1581	
	** * * * ***** ** ***** ** * * * * *		
Mouse	CTCCATTGTCATTCTTTGTGAGGTCTGTCTGCCTTGTGCTTTTGTCCCGGATTAGTCTT	-1176	
Human	CTCCATTGTCATTCTTTGTGAGGTTTGTCTGCCTTGTGCTTTTGTCCCGGATTAGTCTT	-1521	

Mouse	GCTCATGGATGGAGATTTCCAGGCGTGGAGTCTTACAGCTTTTGGCTTCCATTAGGCAAA	-1116
Human	GTTGAAGGATGGAGATTTCCAGGCGTGGAGTCT-ATAGCTTTTGGCTTCCATTAAGTAAA	-1461
	* * * * *	
Mouse	TTGCTAACCTTTTA--AAGGGAAAGTT--TTTTTTTCTTCT-TCATTCCAAAAAGATAG	-1061
Human	ATCGCTAACCTTTTTTTAAGAGAAAGTTAATTTTGCCTGCAGTCATTCCAGAAAAATAG	-1401
	* * * * *	
Mouse	TATGTATG-----AATGTACTTGAAGTT-----TAGTTGCATATGGTAGGT	-1020
Human	TGTACACGGACTAGCTGAATTAATCCACTTAAATGTAACCTAGTTACATATGGTAGAT	-1341
	* * * * *	
Mouse	ATATTAACATGTATCT-----TGGGGGGT	-996
Human	ATGTTAACTCCTATCTGATTTTTTGGATGAACTGTTGTTTTGTTTTAAATAGGAGAT	-1281
	** * * * *	
Mouse	GGGG-----AGACCAGAAACTAC	-978
Human	GAAGTAATGGATTTTTTCCCTCCCTTAAATGGCATAGCTGAGAAAGCCCCGGTACTAC	-1221
	* * * * *	
Mouse	AGGTT-----TTTTTTT-----TTTTTTTTCTAACGAGAAAGG-----	-944
Human	TAATTGAGATGATCTGCTTAAGAGTCTTCATATTCTGAACTCGAAAGGCCTTTATGTTA	-1161
	** * * * *	
Mouse	-ATTGTT-----TTTTA---AAATTTAAATGGCATGCATAGTTGAGAAAG---CCTCTG	-897
Human	TATTATTGCTCGCTTTACGCAACTCCAAATGCTTTCCTATTAAATGAATGTTCTTCTA	-1101
	* * * * *	
Mouse	GTA--CTGAGGTTTGGTT-----ATTGCTATTCTAAATTTGTGTACTCGTCCTTG	-849
Human	GTAAACTGGTGTTAGATTTAAAGAAATCACTCAAGCTTCACCTTGGTAAACTGAACTCAG	-1041
	* * * * *	
Mouse	TTTGGA-----GAGGCATTATTTAATGCTTCTGTTACCCACCG	-811
Human	TTTAAGATTAAGAAACTGATTCGGCCGGGTGCGGTGGCTCACGCCTGTAATTCAGCAC	-981
	* * * * *	
Mouse	TCTGGACTGCTTC-----CTATTTAGATGAAGTTTC---TTTTAGA-----TAACTGG	-767
Human	TTTGGGAGGCCCGGGCGGGCGGATCACCTGAGGTGAGGAGTTCAAGACCAGCCTGACTAA	-921
	* * * * *	
Mouse	TGTT-----AGGTTGGAAGAAACCACCCAAGTGT-----T	-737
Human	TATGGTGAAACCCCGTTTCTACTAAAAATACAAAAAATTAGCCGGGTGTCGTGGCTTGT	-861
	* * * * *	
Mouse	ACCTTTGGT-----AAAAT---GAACT---GAGTTG	-712
Human	GCCTGTAGTCCAGCTACTCCGAGTCTGAGACAGGAAATGCTTGAACCCGGGAGGCG	-801
	* * * * *	
Mouse	AAGATT-----	-706
Human	GAGGTTGCAGTATGCCGAGATCGCTGCAC'TTCAGCCTGGGCGACAGAGCGAGACTCCGTA	-741
	* * *	
Mouse	-----AAGAACTGGTTTTAAACAGACCCTTTGGCCTCTGG	-670
Human	AAAAAAAGAAAAAAAAAAAAATAAAAGAACTGGTTTCAAATTTGGCCCTTTGGCCTCTGG	-681
	* * * * *	
Mouse	AGCTAATTCAAATGTAACCTC-----CCACCCCTTTCTCTTCAGATCAATTAAG	-618
Human	AGCAAATTCAAATGTAACCTCTCCCAACCCCTTCTCTTCTTCAGATTAATTAAG	-621
	* * * * *	

		EXON 2	
Mouse		AAGAATGAGCTGTAATCCCTGAAGATAAACTGGACAGCTTTTTCATTTAGTATATGTAT	-558
Human		AAGAATGAACATAATCCTTGAAGATA--ACTGGGCAATTTTAAAGTCGGAGGCTGT-T	-564
		***** *	
Mouse		ATTTTAAATTATAAAAGTTTCAGGTGGAGTCTTCAGTTTATCAGTACTGAGCAGCAGATCA	-498
Human		CTTACTGGT-GTGAGGATTTACACAC-GTCTTCAGTTTTTCAGCACAGACCAGCAGACCA	-506
		* *	
Mouse		CCATTTTCAGGGACGATACCTGCCT--CTTCTTTTTTGGTGTCTTGGC---AAAAATT	-444
Human		TCATTTTATAGAGGAAATACCTCCTCTGCCCTCCTTTTTGGTTTCCTTGGTGGTAAAGATT	-448
		***** *	
Mouse		AAACCCA-TTGCATCATTGTAACCTCATGTTTGGGTGTAG-TTTCAGGATACA-GTCATGG	-387
Human		AAATTTGGTTGCATCATTTTGACTTGTGTTTGGTCTAGATTTTATGGCACAAGGAATGG	-388
		*** *	
Mouse		TATAAACTTCCACATGCTTTGCTTAAAAAACAACCAACCAAACTCAACCATTACACC	-327
Human		CATAAACTTTTCATGTGTTTTGGTTAAAA-----CAAACCAGACCATTGCATT	-340
		***** *	
Mouse		GAACTTTGGACTTCTTGAATTGAG-----CTTTATTTTAATATAAATACCTGAAG	-277
Human		GA-CCCTGGACATCTTTAATTGAGAAATTGGTAACCTTTATTTTAATATGTATATCTGAAG	-281
		* *	
Mouse		TGATTTTCT---CAGAAGTGTGCTTACGATTATATTCTTATTGTTATTAGAAGCAAACA	-220
Human		AATTCAAGAAACAAAGGCATCTCAGAGGTGTGCCTCTTTCTTTATTATTAG-AGGCA	-222
		* *	
Mouse		AAACTGAGAAGTTTGTAGCATTTTCAATTA---TATCTTAAAGTACAGCTA-AACCAAA	-164
Human		AAAC-GAACAATTTTATAGGATTTGTAGTGAAATTATACCAGATTATAAGGAGAACCAAA	-163
		**** *	
Mouse		ACAGAGTTGTACAATCTATTGAGTAAGAAAATTCATCTTTGGAAAGTAAACATTTCTAAG	-104
Human		ACTAAGTCGCAAAATTTATTAATTTAAGGGGCTCTCGCTTTGAAAGTTTGAGAG--TAAG	-105
		** *	
Mouse		TGAGTCATAAGTATTTGTATACATTC---CCCTTTTCTCTTCAGATTAGGAACTGAGAA	-48
Human		TTA-CGATAGGCATTTGTATCCATTCATTACTTCTCTTTCAAATAAGCAACTAAATA	-46
		* *	
		START CODON	
Mouse		CAACATGCCAGTTGCAGACTTAATCGTTTAAAGCACCAGTGTGGCCATGGAACCTCCA	14
Human		GAA-ATGCTAATCTCAGACTTAATTATTTAACAGAAGAGTGTA-CCATGGAAACCTCCA	14
		* *	
Mouse		GTCTAAGTTCTCCTTAGTTTCAGGGTTCAAATAAAAAGCTGAACGGCATGGAGGATGATGG	74
Human		GACAAATTTCTCCTTGGTTTCAGGGCTCAACTAAAAAAGTGAATGGGATGGGAGATGATGG	74
		* *	
Mouse		CAGTCCTCCTGTGAAAAAA	93
Human		CAGCCCCCAGCGAAAAAA	93
		*** * * * * * * * * * *	

Figure S4

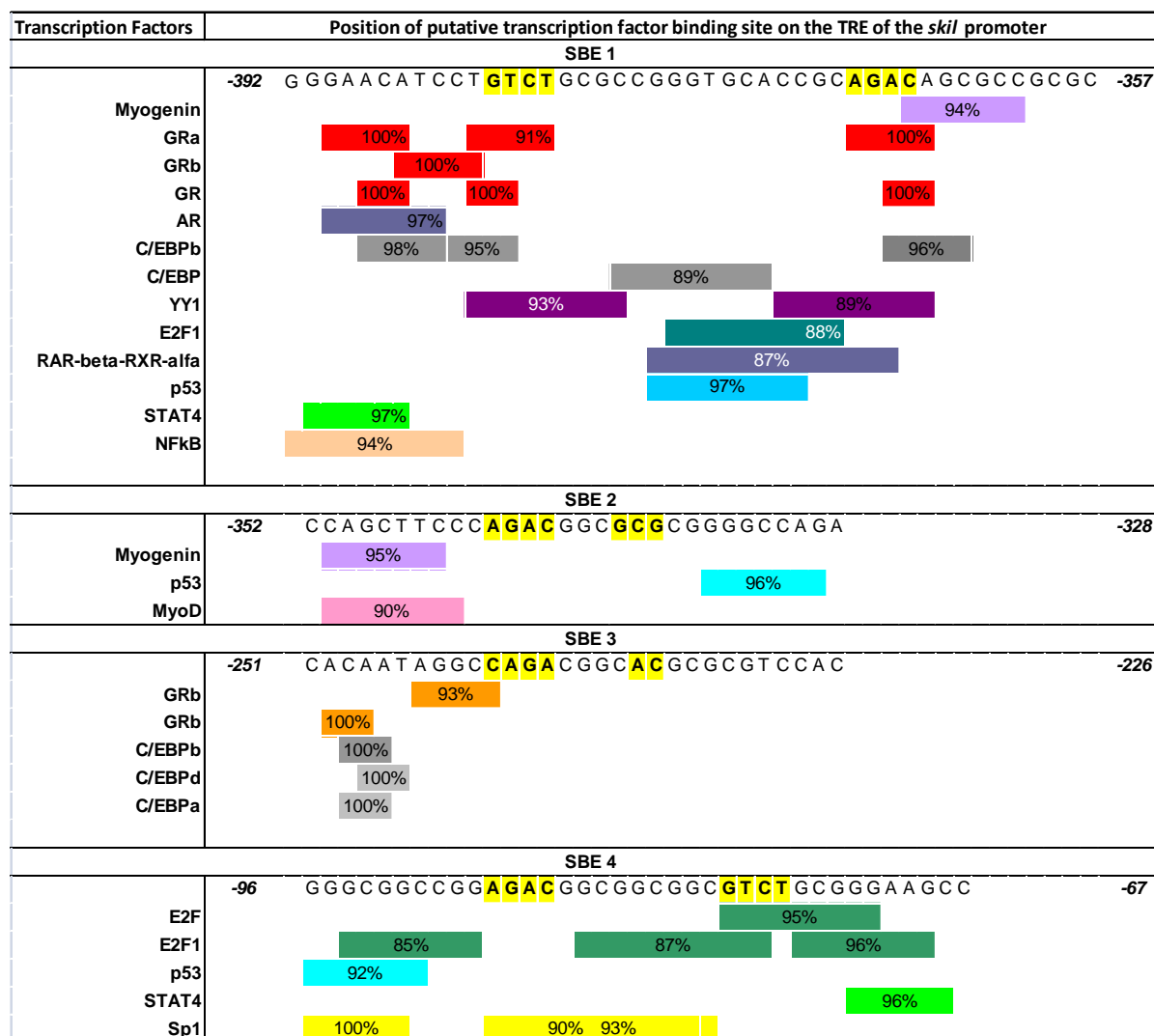
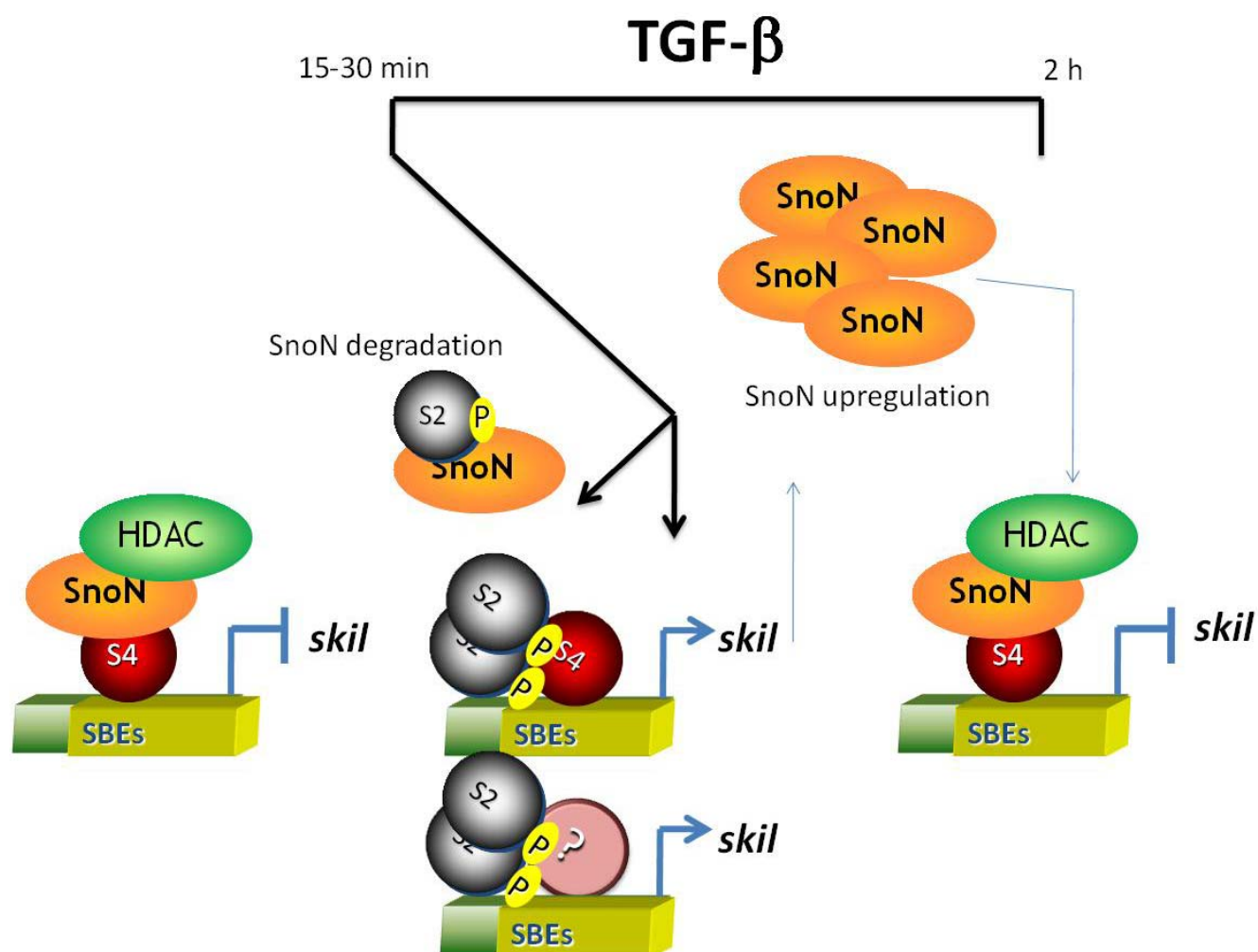


Figure S5



Supplemental Table 1. Websites of the softwares used for bioinformatic study

Program	Websites
GenBank	http://www.ncbi.nlm.nih.gov/genbank/
Genomatix	http://www.genomatix.de/
Softberry FPROM	http://www.softberry.ru/
FirstEF: First-Exon and promoter prediction program for human DNA	http://rulai.cshl.org/tools/FirstEF/
DBTSS: Database of Transcriptional Start Sites. Release 7.0	http://dbtss.hgc.jp/
ALGGEN-PROMO	http://alggen.lsi.upc.est/
GPminer: Gene Promoter miner	http://gpminer.mbc.nctu.edu.tw/
EMBOSS CpGPlot/CpGReport/Isochore	http://www.ebi.ac.uk/Tools/emboss/cpgplot/
CpG Island Searcher	http://www.uscnorris.com/cpgislands2/cpg.aspx
Aspic: Alternative splicing prediction	http://t.caspur.it/ASPIC/

Supplemental Table 2. Oligonucleotides used for PCR

Target		Primer sequence 5' - 3'	Position	Size (bp)
hSnoN	Sense	ctt ctg ctg tat ccc agt cta	nt 1950–1970	308
	Antisense	tca caa gaa gcg gag atg aag c	nt 2258–2237	
hSmad7	Sense	gcc ctc tct gga tat ctt ct	nt 843-862	319
	Antisense	gct gca taa act cgt ggt ca	nt 1162-1143	
β-actin	Sense	ggg tca gaa gga ttc cta tg	nt 222–241	237
	Antisense	ggt ctc aaa cat gat ctg gg	nt 459–440	
skil SBEs (408)	Sense	cgg ggt acc tgg gcg act aga gga agg aa	nt -3100– -3081	430
	Antisense	cgc gag ctc acg tca ctg cgg aga ca	nt -2710– -2692	
skil SBEs (648)	Sense	cgg ggt acc tgg gcg act aga gga agg aa	nt -3100– -3081	648
	Antisense	ggg gag ctc tcg acg agc ttt gtg aat tcc cc	nt -2474– -2451	
pGL3/skilSBEs (408)-Luc (for ChIP on plasmid assay)	Sense	cta gca aaa tag gct gtc cc	nt 2499–2518	630
	Antisense	ctt tat gtt ttt ggc gtc ttc ca	nt 281–303	
A	Sense	caa tag gcc aga cgg ca	nt -2887-- -2871	A+E=380
B	Sense	gga gac ggc ggc ggc gtc t	nt -2738-- -2722	B+E=220 B+F=839 (cDNA) 2776(genomic)
C	Sense	gcc gag gac tcg gtg ac	nt -2710-- -2691	C+E=170
D	Sense	atg tgt gtg ggg ttc gga gc	nt -2646-- -2628	D+E=130
E	Antisense	att gcc cag tta tct tca agg att ata gt	nt -613-- -587	
F	Antisense	cctga acc aag gag aaa ttt gtc tgga gg	nt +9 - +37	

Supplemental Table 3. Oligonucleotides used for SnoN site-directed mutagenesis

Mutant name	Amino acid substituted	Function affected	Primer sequence 5' - 3'
<u>DmSnoN</u> (Δ S2/S3/S4)	Substitution of SLKH (aa 82-85) for GVAA, and Substitution of W315 aa residue for the E315 aa residue	Interaction with S2, S3 and S4	5'CTTTAAATTTAAACCCCgGTgTGgcAgcCA CGCTGGCACAATTCC3' and complementary primer on pCIneo/HA-SnoN (wild type) were used to generate the mutant SnoN(Δ S2/S3). Then: 5'CCCCTGACAAGAGAACTTGCCATgaGGG CTTTGAGTCAGCCAAG3' and complementary primer on mutant SnoN(Δ S2/S3) were used to generate the mutant DmSnoN (Δ S2/S3/S4).
<u>UBmSnoN</u> (KK437, 446AA).	Lysines 437 and 446 were changed for alanines.	Lysines important for ubiquination and degradation of SnoN	5'CCTCCAAACAGTCAGAAAgGCCTCATGA GAGTAGTC3' and complementary primer on pCIneo/HA-SnoN (wild type) were used to generate the mutant SnoN(K437A). Then: 5'GAGAGTAGTCAGCATCAAAGAACAGTGT CTTACCC3' and complementary primer on mutant SnoN(K437) were used to generate the mutant UBmSnoN (KK437, 446AA).