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GROWTH-DEPENDENT REPRESSION OF HUMAN ADENINE NUCLEOTIDE TRANSLOCASE-2 (ANT2) TRANSCRIPTION: EVIDENCE FOR THE PARTICIPATION OF SMAD AND SP FAMILY PROTEINS IN THE NF1-DEPENDENT REPRESSOR COMPLEX.

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Running title: NF1 interacts with Smad proteins

SUMMARY

Nuclear factor 1 (NF1) binds to two upstream elements of the human adenine nucleotide translocator-2 (ANT-2) promoter and actively represses expression of the gene in growth-arrested diploid skin fibroblasts (Luciakova, Barath, Poliakova, Persson and Nelson 2003. J Biol Chem 278:30624-30633). Chromatin immunoprecipitation and co-immunoprecipitation analysis of nuclear extracts from growth-arrested and growth-activated diploid cells, demonstrate that NF1, when acting as a repressor, is part of a multimeric complex that also includes Smad, and Sp-family proteins. This complex appears to be anchored to both the upstream NF1-repressor elements and the proximal promoter, Sp1-dependent activation elements in growth-arrested cells. In growth-activated cells, the repressor complex dissociates and NF1 leaves the promoter. As revealed by co-immunoprecipitation experiments, NF1/Smad4/Sp3 complexes are present in nuclear extracts only from growth-inhibited cells, suggesting that the growth-state dependent formation of these complexes is not an ANT2 promoter-specific event.

Consistent with the role of Smad proteins in the repression complex, TGF- β can fully repress ANT2 transcription in normally growing fibroblasts. Finally, pull-down experiments of *in vitro* transcribed/translated NF1 isoforms by GST-Smad and GST-Smad MH fusion proteins indicate direct physical interactions between members of the two families. These findings suggest a possible functional relationship between the NF1 and Smad proteins that has not been previously observed.

Key words: adenine nucleotide translocator-2, NF1, repression, transforming growth factor-1, human diploid fibroblasts, protein interaction

INTRODUCTION

The Nuclear factor 1 (NF1) transcription factors are encoded in four genes (*Nf1-a*, *Nf1-b*, *Nf1-c* and *Nf1-x*) [1] that are expressed as a large number of splice variants [2-4]. The expressed forms of NF1 bind to DNA as homo- or hetero-dimers [5, 6], thus creating the potential for a large and varied transcriptional network in which NF1 family members activate or repress transcription depending on promoter and cellular context [1]. Many genes are dependent upon NF1 for proper and optimal expression, and NF1 targets the promoters of genes with widely varying functions (reviewed in [1]). NF1 has also been implicated in several growth [7, 8] and developmental [9-11] processes, but never in a central regulatory capacity.

We recently described a unique role for NF1 as an active repressor of gene expression in serum-starved cells [12] using the human adenine nucleotide translocator-2 (ANT2) gene as a model [13]. ANT proteins, which play an important role in maintaining cellular energy homeostasis by catalyzing the exchange of cytosolic ADP for mitochondrial ATP, are encoded in four genes [14]. The ANTs have also been implicated as both positive and negative regulators of apoptosis [15].

ANT2 differs from the other ANT isoforms in that its expression is regulated by the growth- and differentiation-states of cells. In general, ANT2 expression is repressed in growth inhibited [12, 16, 17] or differentiating [17-19] cells. Furthermore, reactivation of growth-inhibited cells activates ANT2 expression on the same time scale as the

immediate-early genes [17], suggesting a functional role in the early events of cell growth. In spite of this, the importance of growth-modulated ANT2 expression remains unclear.

Growth-arrest repression of ANT2 is mediated through NF1 bound to two upstream repressor elements (Go-1 and Go-2) [12, 16]. The Go-1 and -2 repressor elements are occupied by NF1 only in the growth-arrested state, and are required and sufficient for NF1-mediated transcriptional repression [12]. Since ANT2 expression is maintained by Sp1 bound to two juxtaposed Sp1 activation-elements (AB boxes) in the proximal promoter [20], NF1 either directly or indirectly regulates the activity of Sp1.

In the present work, we demonstrate that, when acting as a growth-dependent repressor, NF1 is a component of a protein complex that includes members of the Smad- and Sp-family proteins. This repressor complex contacts both the upstream NF1-binding repressor elements and the proximal promoter Sp1-dependent activation element. In growth-activated cells, NF1 leaves the promoter and the multimeric repressor complex dissociates. The presence of Smad proteins in the NF1 repressor complex, together with our observations that NF1 and Smad proteins interact physically, and that transforming growth factor-beta (TGF- β) represses ANT2 transcription in normally growing cells, suggest a functional collaboration between NF1 and Smad that has not previously been observed.

EXPERIMENTAL

Cell culture. Human primary diploid foreskin fibroblasts in passages 7-20 were grown as described [12]. To arrest cell growth, cells were washed twice with phosphate-buffered saline and incubated for 48 h in serum free media. For growth-activation, serum starved cells were grown an additional 24 h in the presence of 10% serum.

Expression plasmids. The GST-Sp1, GST-Smad2, -Smad3, -Smad4 and the GST-Smad4 MH1 plasmids were generously provided by Dr. A. Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). The Smad2 MH1 domain was prepared by amplifying the domain from amino acid 1 to 251, and the MH2 domain was prepared by

amplifying the domain from amino acid 251 to 467. Similarly, the Smad3 MH1 (amino acid 1 to amino acid 199) and MH2 (amino acid 199 to amino acid 425) domains were prepared; and the Smad4 MH2 domain (amino acid 321 to amino acid 552) was prepared. As a template the appropriate full length Smad in pGEX was used. Amplified MH domains were cloned into the pGEX-6P-1 (Amersham Biosciences) and verified by sequencing. cDNAs coding the NF1-A1, -B2, -C2 and -X1 were provided by Dr. M. Imagawa (Nagoya City University, Japan). For *in vitro* transcription/translation, inserts containing the whole coding region of individual NF1s were recloned into the expression vector pETM11 (EMBL, Heidelberg).

Treatment of cells with TGF- β . Cells in complete medium were treated with 1 ng/ml of TGF- β for 48 h [21].

Preparation of nuclear extract. Cells from starved and serum-induced human diploid fibroblasts were harvested, nuclei were prepared [22] and nuclear proteins were extracted as described in [23]. The protein was measured using the Bio-Rad Protein Assay (Bio-Rad). Nuclear extracts were stored at -70°C.

Immunoprecipitation. Antibodies were covalently linked to Protein A-Sepharose using the Seize X Protein Immunoprecipitation Kit (Pierce). Binding, antigen immunoprecipitation and elution of the antigen was performed according to the manufacturer's manual.

SDS-PAGE and Western blot analysis. Eluted immunoprecipitated samples were precipitated for 20 min on ice in 10% trichloroacetic acid, followed by a 15-min centrifugation at 10 000 x g and two washes with ice-cold acetone. Samples were air-dried, dissolved in sample buffer and separated by 10% SDS-PAGE [24]. After electrophoresis, samples were electroblotted onto the Hybond ECL membrane (Amersham Biosciences). Membranes were incubated with appropriate antibodies and developed with SuperSignal West Pico Chemiluminiscent Substrate (Pierce).

Chromatin immunoprecipitation. Chromatin immunoprecipitation from growth-arrested and growth-induced human diploid cells was performed as in [25], except that 100 μ l of Protein A-Sepharose was used instead of *Staphylococcus aureus* cells. Immunoprecipitation was performed with either 2 μ l of antiserum against NF1 (#8199, kindly provided by Dr. Tanese), or 2 μ g of antiserum against Smad4 (*sc-7154*, Santa

Cruz Biotechnology), Smad 1/2/3 (*sc-7960*, Santa Cruz Biotechnology), Sp1 (*sc-59*, Santa Cruz Biotechnology), Sp3 (*sc-644*, Santa Cruz Biotechnology), Smad 2 (*sc-6200*, Santa Cruz Biotechnology) and p300 (*sc-585*, Santa Cruz Biotechnology). Amplification of immunoprecipitated DNA fragments (2 μ l) was performed using the primers -931 (5'-AGTTCTTAACCTTCCTAAGCCTC-3') and -666 (5'-CAAGTGAATG CCTCACTCTTCC-3') covering the GoR; and -151 (5'-TCCACGACTCCTCCTCCTGCGAGC-3') and +39 (5'-CTGCAGGA CGGGACTGG GCCGGGAAC-3') covering the AB boxes. PCR was performed for 26-30 cycles, with 45 s of denaturation at 94°C, followed by 45 s of annealing at 60°C and 45 s of extension at 72°C. The last step included extension for 10 min at 72°C.

In vitro and bacterial expression of proteins. *In vitro* expression and radiolabeling of proteins was performed using the Promega coupled transcription/translation system (TNT). Plasmids were transcribed using the T7 RNA polymerase according to the manufacturer's manual. The quality of translated proteins was verified by SDS-PAGE. Glutathione S-transferase (GST) fusion proteins were expressed in *E. coli* strain BL21, and purified as described in [26]. Solubilization and purity of the expressed proteins were monitored by SDS-PAGE.

Pull-down experiments. 1 μ l of the ³⁵S-labeled NF1s, obtained by *in vitro* transcription/translation, were incubated with 1 μ g of GST or GST-Smad2, -Smad3, -or Smad4 bound to glutathione-Sepharose beads (Pharmacia). Binding of NF1 to GST fusion proteins was done in 50 mM T-Cl, pH 8, 50 mM KCl, 0.02% Tween 20, 0.02% BSA, 0.5 mM DTT and washing of bound proteins was performed in 50 mM T-Cl, pH 8, 50 mM KCl, 1% Triton-X100, 0.02% BSA, 0.5 mM DTT. Associated proteins were separated by SDS-PAGE and visualized on Typhoon Amersham Phosphoimager.

RNA purification and RT PCR. RNA was isolated using the acidic guanidinium thiocyanate/phenol/chloroform extraction [27]. RNA (1 μ g) was reverse transcribed to generate the first strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer's instructions. 1/15 of the cDNA was used as template in PCR (25 μ l) using primers for the ANT2 and ANT3 coding region, respectively. The 5'-primer was common for all three ANT isoforms and covered nts +160/+181 in ANT2 and nts +184/+195 in ANT3 (5'-

GGGTCAAGCTGCTGCTGCAGG-3'), respectively. The 3'- primer of ANT2 covered nts +509/+532 (5'-CGGAATTCCCTTTCAGCTCCAGC-3'), and the 3'-primer for ANT3 covered nts +443/+464 (5'-CCGGCCGCACCGCCGGAGGC-3'). PCR reaction was performed for 28 cycles with 1 min of denaturation at 94°C, 30 s annealing at 63°C and extension for 1 min at 72°C. PCR products were separated on 2% agarose and stained with ethidium bromide.

RESULTS

The ANT2 promoter contains 3 repressor elements that modulate the activity of Sp1 on the AB box (Fig 1). However, repression of ANT2 transcription in growth-arrested cells (either by serum starvation or by growth to confluence) is mediated solely through NF1-binding elements (Go-1 and Go-2) in the GoR [12, 16]. The ANT2 promoter also contains a number of putative Smad binding elements (5'-GTCT-3', or 5'-AGAC-3') that are distributed around and between the Go-1 and Go-2 elements, between the two Silencer elements, and immediately upstream of the Sp1 activating elements (Fig 1). This distribution, together with data suggesting a link between NF1 and the TGF- β signaling system [28], raised the question if Smad proteins could play a role in NF1-dependent repression of ANT2 in serum-starved cells.

To address this issue, we first carried out a series of co-immunoprecipitation experiments to determine if NF1 and Smad proteins interact, either directly or as components of a common multimeric complex. Experiments were done on nuclear extracts from serum-starved (growth-arrested) and serum re-fed (growth-activated) diploid fibroblasts (Fig 2). The existence of Smad/NF1 nuclear complexes could be demonstrated by co-immunoprecipitation with antibodies to both Smad4 (Fig. 2, left panel) and NF1 (Fig 2, right panel). Unexpectedly, NF1/Smad4 complexes were detected in nuclear extracts predominantly, if not exclusively, in serum-starved cells (Fig 2, lane S vs. I). Thus, formation of nuclear complexes containing NF1 and Smad protein appears to be growth-state dependent.

To determine if Smad/NF1 nuclear complexes are associated with the ANT2 promoter, chromatin immunoprecipitation (ChIP) analysis was performed on chromatin

isolated from serum-starved and serum re-fed cells. The results show that Smad4 (Fig 3A, upper panel, compare lanes 4 and 5) and NF1 (Fig 3B, lanes 4) are both associated with the Go-2/Go-1 elements in serum-starved cells. By contrast, neither protein is associated with the ANT2 promoter in growth-activated cells, suggesting that release of NF1 is accompanied by release of Smad4. However, Smad4 is detected on the AB box in re-fed cells (Fig 3A, lower panel, lane 5). The growth-activated loss of NF1 and Smad4 from the Go repressor and the appearance of Smad4 associated to the AB activation boxes suggest a re-organization of the repressor complex in growth activated cells.

We also tested for the presence of R-Smads (Smad2/3) [29] on the GoR and AB box elements. ChIP analysis using antibodies to Smad2/3 or Smad2 show that Smad2, and perhaps also Smad3, are associated with both the GoR and the AB box regulatory elements in serum-starved cells (Fig. 4A, lanes 4), but to neither of these elements in growth-activated cells (Fig 4A, lanes 5). Thus, Smad2/3 is associated with both the Go and AB elements, but only in the repressed state. However, co-immunoprecipitation of nuclear extracts (Fig 4B) show that NF1/Smad2/3 complexes are present in the nucleus under all conditions of growth, suggesting that they may not dissociate after leaving the Go repressor element. By contrast, nuclear complexes containing both Smad4 and Smad2/3 appear to decrease in serum-activated cells (Fig 4B, lane I), suggesting that the Smads might dissociate upon growth-activation. Since co-precipitation experiments probe the total population of nuclear complexes, not just the subset of those bound to the ANT2 promoter, identification of Smad/NF1 complexes by this method provides reason to believe that such complexess participate in the regulation of additional genes.

The association of NF1/Smad complexes to the AB box is most probably mediated through the Sp proteins. We, therefore, conducted ChIP analysis of Sp1 and Sp3 binding to the ANT2 promoter in serum-starved and serum-activated cells (Fig 5). Sp3 was included in the analysis since it binds the same element as Sp1, but is the only member of the Sp family that acts as a repressor [30]. Sp1 (Fig 5A, lane 4) and Sp3 (Fig 5B, lane 4) are associated with both the Go element and the AB box elements in serum-starved cells, suggesting that these elements might be linked through a larger repressor complex. Upon serum-reactivation, Sp3 is no longer detected (Fig 5B, lanes 5) and appears to leave the

promoter, while Sp1 remains bound only to the AB boxes (Fig 5A, lower panel, lane 5). Thus, it would appear as though Sp1 and Sp3 are anchored to the Go repressor and the AB activation boxes together with NF1 and Smad2/3 in growth-arrested cells. However, upon re-feeding with serum Sp3 leaves the promoter while Sp1 remains anchored to the AB activation element (Fig. 5, lane 5, lower panels).

To gain further insight into the repressor complex, we investigated the association between the Sp and Smad proteins under different growth states. Sp1 antibodies co-precipitate Smad4 from nuclear extracts prepared from both serum-starved and re-fed cells (Fig 6A), indicating the presence of Sp1/Smad4 nuclear complexes under all conditions of growth. However, a second Smad protein is also co-precipitated by Sp1 antibodies, but only from serum-starved cells (Fig 6A, asteriks). The growth-state dependency of this latter Smad/Sp1 complex suggests a possible role in G0 repressor assembly/disassembly. However, the identity and functional significance of this Smad protein remains unknown.

In contrast to the presence of nuclear Sp1/Smad4 complexes in both serum-starved and serum-refed cells, Sp3/Smad4 complexes are detected by co-immunoprecipitation only in nuclear extracts of serum-starved cells (Fig 6C). Thus, the dissociation of nuclear Sp3/Smad4 complexes appears to be related to the growth repression/activation transition in diploid fibroblasts. This conclusion is strengthened by ChIP analysis showing that Sp3 leaves the AB boxes in growth-activated cells (Fig 5B), resulting in the Sp1-dependent activation of ANT2 expression [20].

Finally, Sp1 antibodies co-precipitate NF1 from nuclear extracts prepared from growth-arrested cells, but not from growth-activated cells (Fig 6B). Thus, as with the Sp3/Smad4 complexes, formation of nuclear Sp1/NF1 complexes in human diploid fibroblasts is growth-state dependent. This conclusion is also consistent with ChIP experiments showing that Sp1/NF1 complexes bind to the ANT2 promoter only in serum-starved, growth-inhibited cells (Fig 3)

p300 interacts directly with Smad proteins [31, 32] and functions as a co-activator. We therefore tested if p300 acts as a co-activator on the ANT2 promoter. Antibodies to p300 co-precipitate Smad4 from nuclear extracts of serum-activated cells, but not from serum-starved cells (Fig 6A). Thus, formation of p300/Smad4 complexes is also growth-

state dependent in diploid fibroblasts, favoring the growth-activated state. However, even though nuclear p300/Smad4 complexes are formed in growth-activated fibroblasts (Fig 6A), a significant increase in p300 binding to the AB activation boxes could not be detected by ChIP analysis (Fig 5A, lower panel). Thus, p300 may not act as a co-activator on the ANT2 promoter. Antibodies to p300 co-precipitated little or no NF1 from nuclear extracts of either growth-arrested or growth-activated cells (Fig 6B), suggesting that the population of nuclear complexes containing both p300 and NF1 is relatively small, if existent, in human diploid fibroblasts. This result supports our conclusions from ChIP analysis that p300 is not associated with the G0 repressor complex (Fig 5A).

The question remains if nuclear NF1/Smad complexes are formed through direct or indirect physical contacts. Smad [31, 33] and NF1 [34] interact directly with Sp1, providing a possible assembly pathway for NF1/Smad-containing complexes. To test for physical interactions, GST fusion proteins (full length Smads or their MH1 or MH2 domains) were used to pull-down *in vitro* translated, radiolabeled full length NF1s. Smad3 and Smad4 interact relatively strongly with the NF1-A and -C (Fig 7, left panel) through their MH1 domain (Fig 7, right panel). Smad3 and Smad4 appear to interact more weakly with full length NF1-B and NF1-X (left panel). However, pull down with the MH domains (right panel) suggests that Smad3 and Smad4 might interact with NF1-X through the MH1 domain. Interactions of Smad2 are less clear. Though it appears to interact as strongly as Smad3 and 4 with full length NF1-A (Fig 7, left panel), this was not confirmed using the MH-domain fusion proteins (Fig. 7, right panel). Taken together, results in Fig. 7 show for the first time a direct physical interaction between the transcription factor NF1 and Smad2, Smad3 and Smad4.

The inclusion of Smad proteins in the Go-repression complex suggests that TGF- β might be a signaling ligand in ANT2 repression. To test this we measured ANT2 transcripts in normally growing diploid fibroblast treated with TGF- β . The results (Fig 8) show that TGF- β prevents ANT2 expression in growing cells, and that the magnitude of the TGF- β inhibitory effect is similar to that obtained by serum removal (Fig 8, ref. [12]. As expected [35], serum starvation does not effect the expression of ANT3 (Fig 8).

DISCUSSION

Expression of the human ANT2 gene is repressed in serum starved cells and in cells grown to confluence [12, 16]. We earlier mapped and identified two upstream promoter elements (Go-1 and Go-2) required for repression [12]. These elements bind NF1, which acts as an active repressor. To gain insight into the mechanism by which NF1 represses Sp1-dependent activation, we studied the proteins associated with the Go and AB box elements in growth-repressed and growth-activated human skin fibroblasts. We show that NF1 bound to the Go elements in growth-repressed cells is part of a larger repressor complex that includes both Smad and Sp-family proteins. Figure 9 summarizes the proteins found by ChIP analysis to be associated with the Go and AB box elements in growth-activated and growth-repressed cells. Complexes containing NF1, Smads2/3, Smad4, Sp1 and Sp3 are associated with both the Go and AB Box elements in serum-starved cells. These data could be explained by the binding of separate and distinct complexes to each set of elements, as depicted in Fig 9. However, it is equally feasible that NF1 and Sp proteins anchored to the Go and AB box elements, respectively, are linked in a repressor complex that includes Smad proteins.

Whatever the nature of the complexes, growth-activation results in an extensive reorganization on the promoter in which NF1 and the associated Smad and Sp proteins leave the Go element. These findings are in accord with our earlier failure to detect protein binding to the Go elements in growth-activated cells using *in vivo* foot printing [12]. Release of the repressor complex from the Go elements in growth-activated cells is also accompanied by reorganization around the AB elements (Fig 9). Sp1 remains on the AB boxes, but is no longer associated with NF1, Smad2/3 or Sp3. Sp1 is, however, associated with Smad4. Reorganization of Sp1 bound to the AB elements in growth-activated cells is also suggested by a shift in the specific nucleotides contacted by Sp1 [36].

NF1 binding to the Go element in the growth-repressed state, and its release in the growth-activated state [12], suggest a central role for this protein in ANT2 repression. However, the mechanism of NF1 recruitment to the promoter and the order in which the components of the repressor complex are assembled is difficult to assess since Sp1

interacts directly with the Smads [31, 33] and NF1 [34], and, as demonstrated in the present study, NF1 also interacts with Smads. The dynamic redistribution of Smads is only one of many possible mechanisms by which assembly of a repressor complex might be regulated, especially as the cytoplasmic/nuclear distribution of Smad proteins is a dynamic process, and some of these proteins are present in the nucleus even in non-stimulated cells [37, 38]. Furthermore, all three proteins can be modified by various combinations of phosphorylation, acetylation or Sumoylation, and are, thus, potential targets for signaling pathways that initiate repressor assembly/disassembly. Despite these complications, our observation that TGF- β induces repression of the ANT2 promoter in proliferating human diploid fibroblasts implies involvement of a Smad-dependent pathway. Furthermore, since TGF- β is released from serum-starved human diploid fibroblasts [39] and is responsible for initiating a senescence response [39], it is a prime candidate as the ligand that initiates ANT2 repression in the absence of serum.

A mechanism of NF1-dependent repression similar to that described here and in our previous studies [12] on the ANT2 promoter was recently reported for the p21Waf1/Cip1 promoter in human skin fibroblasts [40]. With both promoters, NF1 binding represses transcription, whereas release of NF1 is associated with transcriptional activation. Furthermore, Sp3 is associated with NF1 on both promoters in the repressed state, but not in the activated state (discussed below). However, regulation of the two genes by NF1 differs in that repression of ANT2 takes place in growth-arrested human skin fibroblasts, whereas p21 repression occurs in proliferating cells. Thus, NF1, acting as a direct repressor, is able to regulate genes with vastly different growth-related functions in skin fibroblasts.

NF1 has been implicated in a variety of growth-related processes, even though a central role for NF1 in growth regulation has not been established. NF1 represses *gadd153* [7] and activates *p53* [8] expression, both of which have key roles in growth arrest of damaged or stressed cells. Furthermore, NF1-X, together with *c-myc* and *mdm2*, were the only genes identified in a screen performed to identify complementary DNAs that prevent TGF- β -induced growth arrest of mink lung epithelial cells [28]. Finally, expression of NF1 in chick embryo fibroblasts has also been reported to prevent transformation induced by nuclear, but not cytosolic, oncogene products [41]. These

studies, together with the present results demonstrating the existence of NF1/Smad nuclear complexes (Figs. 2 and 4B), suggest that NF1 may have a more general role in regulating growth-related processes than previously appreciated.

NF1-mediated repression of ANT2 described here is reminiscent of the TGF-dependent repression of the *myc* and *Id1* genes which are mediated through Smads cooperating with a transcription factor acting as a co-repressor. *Myc* repression is mediated through the p107 repressor protein which is recruited to a Smad/E2F/DP1 complex [42], and *Id1* repression is mediated via ATF3 in cooperation with Smad3 [43]. In the case of ANT2, assembly of the NF1/Smad complexes on the promoter is also associated with recruitment of a known repressor protein, Sp3. Sp3 is associated with a NF1-repressor complex on both the Go and the AB box elements in growth-arrested cells, and is released from the promoter in growth-activated cells. Similarly, Sp3, but not Sp1, is bound to the NF1-repressed p21Waf1/Cip1 promoter in proliferating skin fibroblasts [40]. These results suggest that Sp3 could be a specific component of the NF1-dependent repression machinery. Sp3, unlike Sp1, is a strong transcriptional repressor [30]. Silencing is dependent on the lysine residue in an IKEE repressor motif, which, when mutated, converts Sp3 into a transcription activator [44]. *In vivo* acetylation of Sp3 activates expression of the TGF- β type II receptor [45] and the SOCS [46] genes, suggesting a possible signaling mechanism for the Sp3 repressor/activator switch. However, the IKEE motif is also Sumoylated, which, like acetylation, eliminates Sp3 silencing and promotes Sp3 activation [47-49]. Thus, the specific role of Sp3 in ANT2 repression remains to be sorted out.

To our knowledge, no structural or functional associations between members of Smad and NF1 families have been reported previously, even though, as discussed above, several reports suggest interfacing between the NF1 proteins and TGF-mediated processes. However, the present study demonstrates clearly: a) that Smad and NF1 proteins interact physically, as shown by *in vitro* pull-down assays, and b) that nuclear complexes of Smad/NF1 are formed and disassembled in human skin fibroblasts in a growth-state dependent manner. These findings, coupled to our observation from ChIP analysis that Smad proteins are components of a NF1 complex that acts to repress ANT2

expression, raise the possibility that NF1/Smad may have a broader function in the regulation of gene expression than heretofore appreciated.

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The authors declare no conflict of interest.

The abbreviations used are: ANT, adenine nucleotide translocator; GoR, Go repressor region; Go-1 and Go-2, Go NF1-binding repressor elements 1 and 2; TGF- β , transforming growth factor- β ; GST, glutathione S-transferase; ChIP, chromatin immunoprecipitation.

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FIGURE LEGENDS

Fig. 1. **The human ANT2 promoter.** Positions of known regulatory regions are placed relative to the transcription start (arrow). Empty boxes represent the Sp1 AB-activation elements. Three repressor regions, the GoR, Silencer and the C box are marked as grey boxes. Black ovals represent putative Smad-binding elements. The Go repressor and the Silencer bind NF1, whereas the C-box binds Sp1.

Fig. 2. **NF1 and Smad4 co-immunoprecipitate in growth-arrested cells.** To arrest cell growth, cells were washed twice with phosphate-buffered saline and incubated in serum free media for 48 h. To reactivate growth, serum starved cells were grown an additional 24 h in the presence of 10% serum. Nuclear extracts (500 µg) from serum-starved (S), and serum-induced (I) cells were isolated and immunoprecipitated (IP) with antibodies against Smad4 or NF1. Protein eluted from the ProteinA-Sepharose were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with specific antibodies (WB for western blots). Arrows indicate the MW standards. A representative picture of four independent experiments is shown.

Fig. 3. **NF1/Smad4 repressor complex binds to the GoR only in growth-arrested human diploid cells.** Chromatin immunoprecipitation was carried out on formaldehyde-crosslinked cells deprived of serum for 48 h (lanes 4), or serum-deprived and subsequently grown in the presence of serum (lanes 5). Chromatin was isolated and immunoprecipitated with: **A)** anti-Smad4 or **B)** anti-NF1 antibodies. DNA was amplified with primers covering the Go-2/Go-1 repressor elements (nts -931/-666) and the AB box Sp1-activation elements (nts -151/+39). Primer amplification was also carried out on: a negative control containing no template (lanes 1), template isolated from chromatin which was not immunoprecipitated (lanes 2), chromatin immunoprecipitated with an unrelated antibody, anti-F1-ATPase (lanes 3), or total chromatin (lanes 6). A 100-bp gene ruler (MBI Fermentas) marker is also shown (M). A representative picture of four independent experiments is shown.

Fig. 4. Smad2 and Smad 3 form a complex on the GoR. **A)** Chromatin immunoprecipitation was carried out on formaldehyde-crosslinked serum-starved (lanes 4) or serum-activated (lanes 5) cells. The primers used and the definition of the remaining lanes are described in Fig 3. **B)** Immunoprecipitation of Smad2/3 with antibodies to NF1 or Smad4. Nuclear extracts (500 µg) from serum-starved (S) and serum-induced (I) cells were isolated and immunoprecipitated (IP) with antibodies against the indicated proteins. Smad2/3 detected by western blotting is indicated by an arrow on the right side. Arrow on the left side show the MW standards. A representative picture of two independent experiments is shown.

Fig. 5. Sp1 and Sp3 are associated with the Go repressor. **A)** Chromatin immunoprecipitation was carried out on serum-starved (lane 4 and 6) and serum-induced (lane 5 and 7) diploid fibroblasts using antibodies against Sp1 or p300. **B)** Chromatin immunoprecipitation was carried out on starved (lanes 4) and induced (lanes 5) cells using the antibodies against Sp3. DNA was amplified with primers covering the GoR (Go-2/Go-1) and the AB box (AB box). Lanes 1-3 are as described in Fig 3, lanes 8 (A) and 6 (B) is total chromatin. A representative picture of three independent experiments is shown.

Fig 6. Interactions of Sp proteins and p300 with the NF1/Smad complexes. **(A and B)** Nuclear extracts (500 µg) from serum-starved (S) and serum-induced (I) cells were immunoprecipitated (IP) with antibodies against Sp1 or p300. Proteins resolved by 10% SDS-PAGE were probed with Smad4 or NF1 antibodies (SantaCruz Biotechnology). Smad4 or NF1 detected by western blotting are indicated by an arrow on the right side. Arrows on the left side show the MW standards. **C)** Nuclear extracts from serum-starved (S) and serum-induced (I) cells were immunoprecipitated with antibodies against NF1 and Smad4, and then probed with antibodies against Sp3. A representative picture of two independent experiments is shown.

Fig. 7. Smad proteins physically interact with transcription factor NF1. GST-Smad2, -3 and -4 and their individual MH domain fusion proteins were expressed as

described in Materials and Methods. GST-Smad protein (1 μ g) was bound to glutathione beads and incubated with radiolabelled NF1 isoforms produced by *in vitro* transcription/translation in rabbit reticulocyte lysates. (5% input), input of the 35 S-labeled *in vitro* synthesized NF1 proteins. (GST), empty vector carrying GST alone. Left, NF1 isoform synthesized *in vitro*. A representative picture of four independent experiments is shown.

Fig. 8. TGF- β inhibits expression of ANT2. Total RNA (1 μ g) from human diploid foreskin fibroblasts was reverse-transcribed and amplified with ANT2 and ANT3 specific primers. RNA was isolated from cells grown in the absence of serum for 48 h (S), from serum starved cells subsequently induced for 24 h with 10% serum (I), and from cells grown for 48 h in complete medium and in the presence of TGF- β (1 ng/ml). A representative picture of three independent experiments is shown.

Fig. 9. The role of NF1, Smad and Sp proteins in growth repression/activation of ANT2 gene. A complex containing NF1, Smad and Sp proteins binds to the GoR and Sp1 promoter elements in serum-starved cells. Upon activation of cell growth, NF1-Smad2/3 and Sp3 are released from the Go element, leaving a complex of Sp1 and Smad4 bound to the proximal Sp1 transcription-activation elements (AB box). The repressor elements of the GoR and the C box (see the main text) are depicted by grey boxes. The filled ovals are putative Smad binding elements. An arrow indicates the transcription initiation site, a cross indicates transcription inhibition.

Fig. 1. Luciaková et al.

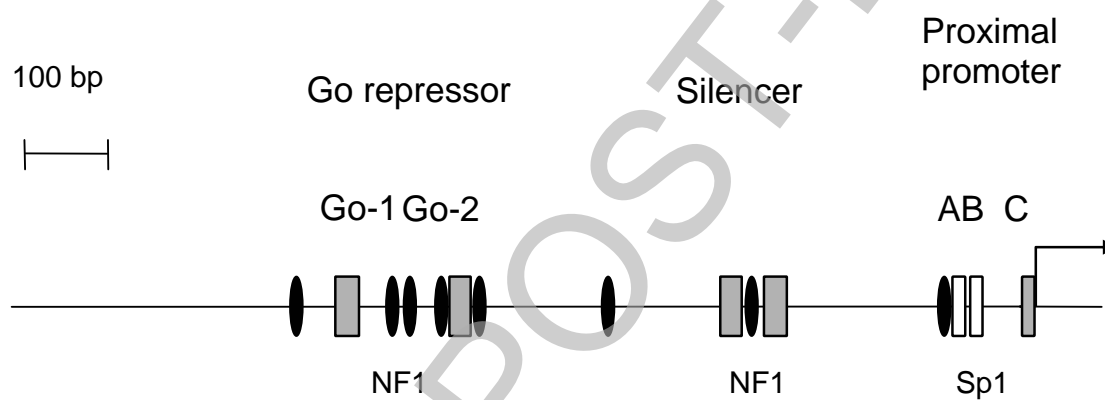
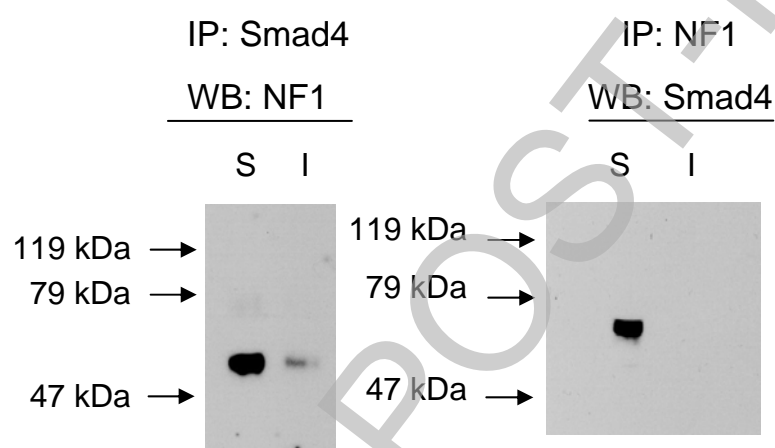


Fig. 2, Luciakova et al.



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Fig. 3, Luciakova et al.

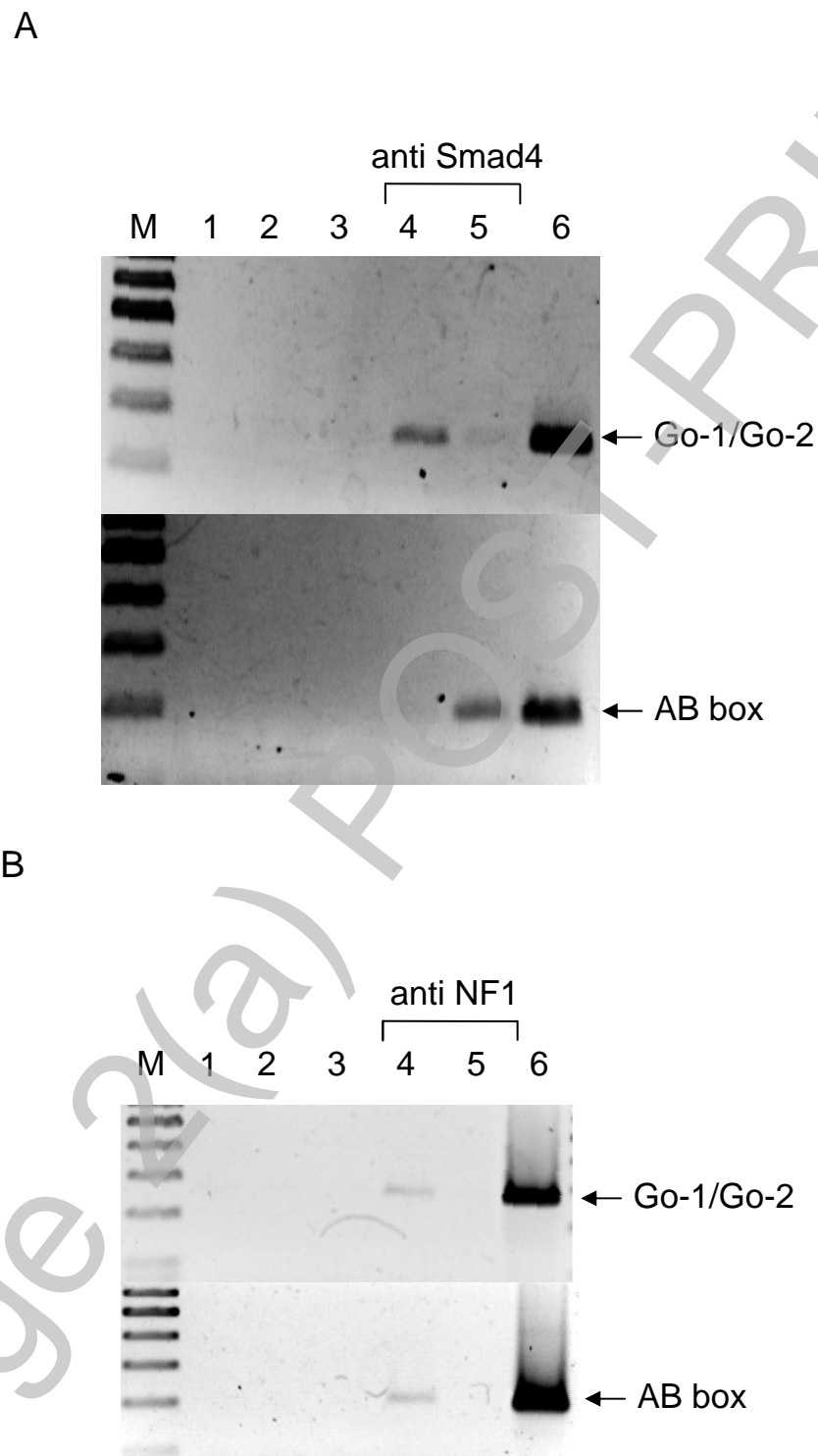
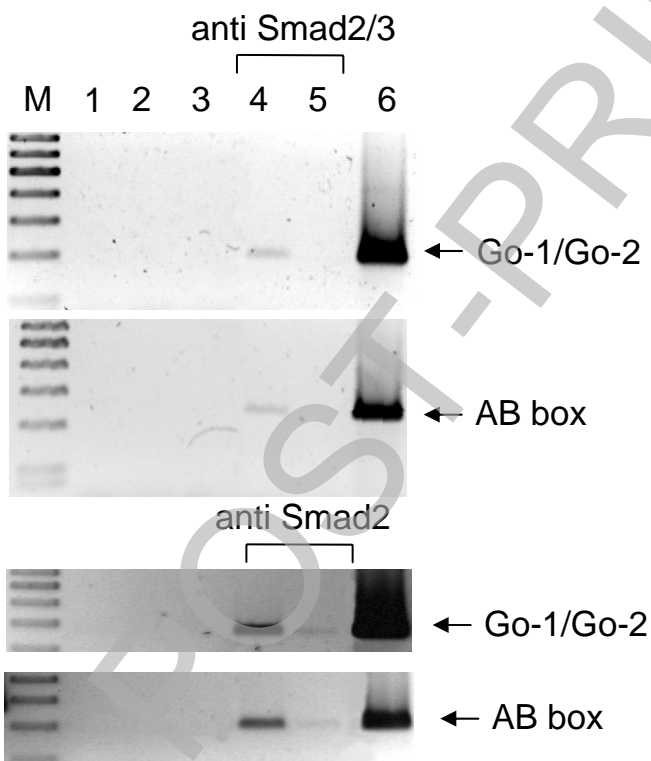


Fig. 4, Luciakova et al.

A



B

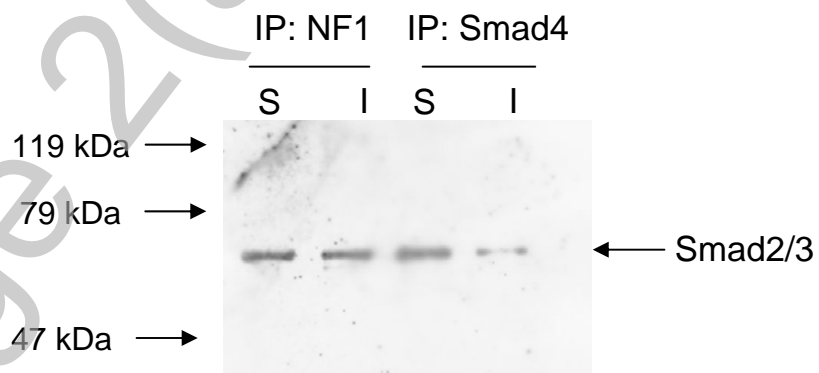
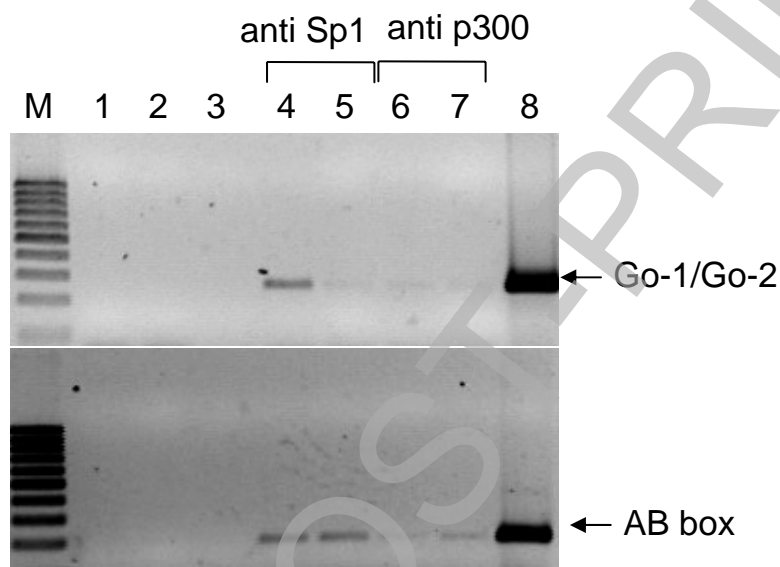
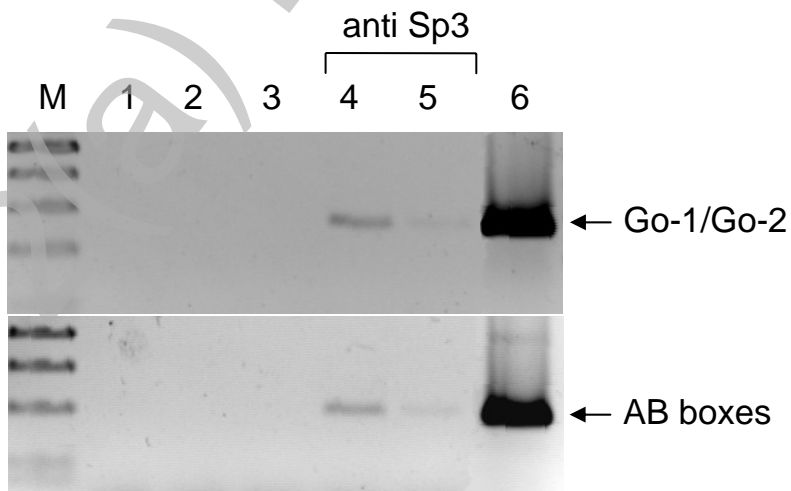


Fig. 5, Luciakova et al.

A

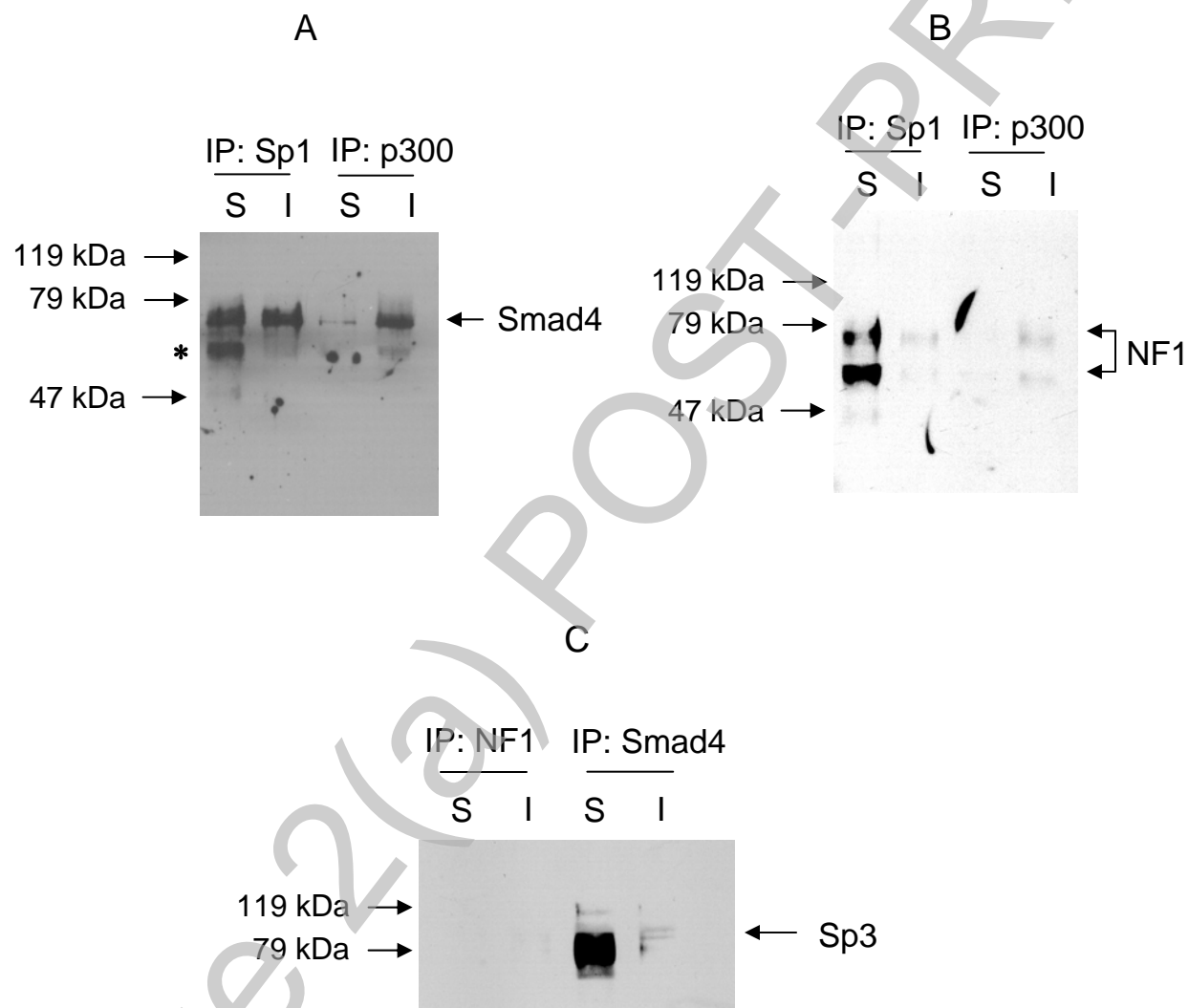


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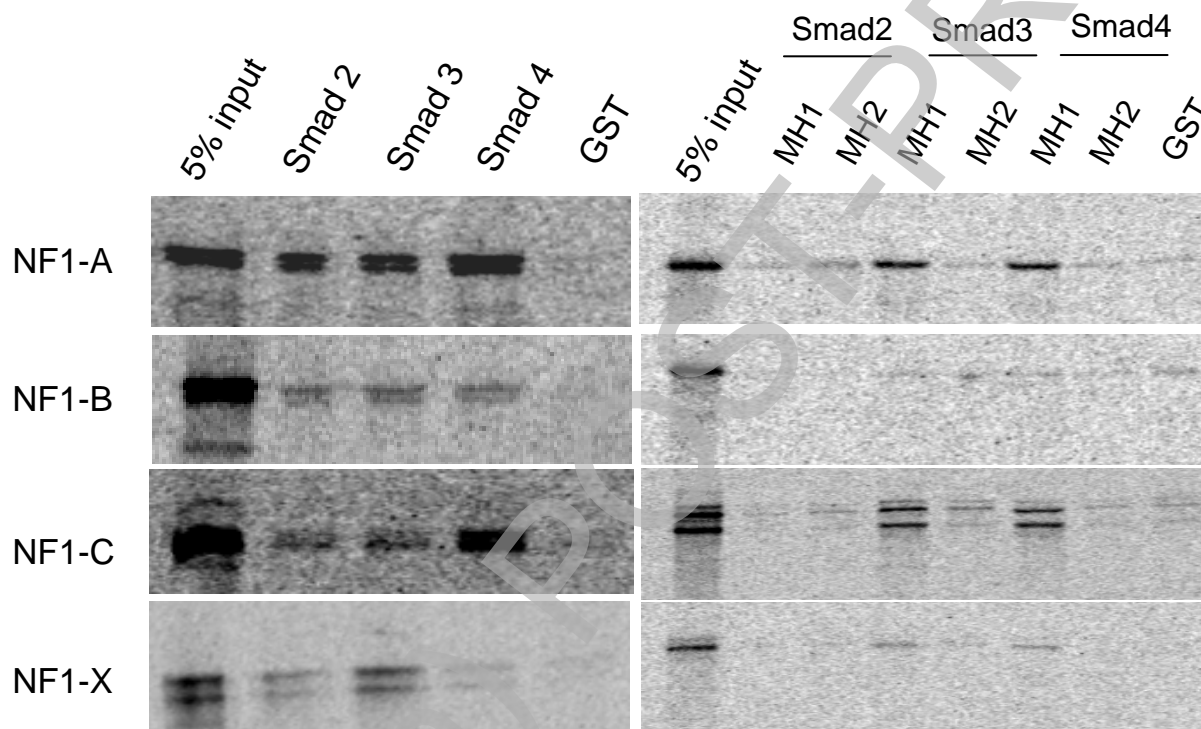
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Fig. 6, Luciakova et al.



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Fig. 8, Luciakova et al.

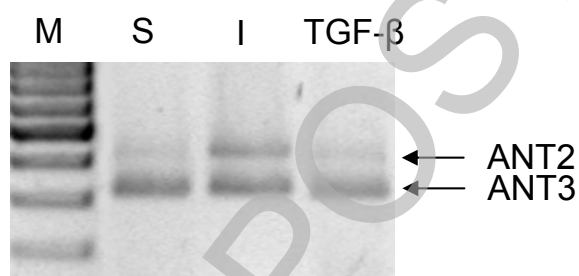


Fig. 9, Luciaková et al.

