Published in final edited form as:

Cell Signal. 2012 April ; 24(4): 922–930. doi:10.1016/j.cellsig.2011.12.015.

SnoN oncoprotein enhances estrogen receptor- α transcriptional activity

Arja M. Band^a and Marikki Laiho^{a,b,*}

^aMolecular Cancer Biology Program, Biomedicum Helsinki and Haartman Institute, University of Helsinki, Helsinki, Finland ^bSidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, MD, United States

Abstract

Estrogen receptor- α (ER α) and transforming growth factor-beta (TGF- β) signaling pathways are essential regulators during mammary gland development and tumorigenesis. Ski-related novel gene (SnoN) is an oncoprotein and a negative feedback inhibitor of TGF- β signaling. We have previously reported that low expression of SnoN in ER α positive breast carcinomas is associated with favorable prognosis (Zhang et al. *Cancer Res.* (2003) 63, 5005–5010). Here we have studied the mechanism of a possible cross-talk between ER α and SnoN. We find that SnoN interacts with the estrogen-activated form of ER α in the nucleus. SnoN contains two highly conserved nuclear receptor binding LxxLL-like motifs and we show that mutations in these motifs reduce the interaction of SnoN with ER α . Over-expression of SnoN enhanced the transcriptional activity of ER α in estrogen response element (ERE)-reporter assays, augmented the expression of several ER α target genes and increased the proliferation of MCF7 breast carcinoma cells in an estrogen-dependent manner. Chromatin immunoprecipitation demonstrated that SnoN interacts with ER α at the *TTF1* (pS2) gene promoter. Conversely, silencing of SnoN reduced both ERE-reporter activity and the expression of ER α target genes in MCF7 and T-47D breast cancer cells. Histone deacetylase inhibition increased the level of SnoN and SnoN-dependent enhancement of ER α -dependent transcription and SnoN supported the recruitment of p300 histone acetylase to ER α . This study reveals a novel mechanism that interconnects ER α and TGF- β signaling pathways by SnoN. Accordingly, the results indicate that high SnoN level promotes ER α signaling and possibly breast cancer progression.

Keywords

SnoN; Estrogen receptor- α ; Transforming growth factor β ; Transcription; Ski

© 2012 Elsevier Inc. All rights reserved.

*Corresponding author at: Department of Radiation Oncology and Molecular Radiation Sciences, The Johns Hopkins University School of Medicine, 1550 Orleans Street, Room 444, Baltimore, MD 21231, United States. Tel.: +1 410 502 9748; fax: +1 410 502 2821. mlaiho1@jhmi.edu (M. Laiho).

Supplementary materials related to this article can be found online at doi:10.1016/j.cellsig.2011.12.015.

Conflict of interest

The authors declare no conflict of interest.

Author contribution

A.M.B. and M.L. designed the research and analyzed the data, and A.M.B. performed the experiments. The manuscript was written by A.M.B. and M.L.

1. Introduction

Transforming growth factor- β (TGF- β) is a major regulator of essential cellular processes including proliferation, differentiation, migration and apoptosis. It has a dual role in tumor progression. Due to its anti-proliferative effects and maintenance of tissue homeostasis, it acts as a tumor suppressor, but for unknown reasons, TGF- β signaling may convert to a tumor promoter. In such case, TGF- β promotes epithelial-to-mesenchymal transition, metastasis, and acts as an immune suppressant [1]. TGF- β initiates the activation of its signaling pathway by binding to its receptors at the cell surface. This leads to activation of the receptor complex and phosphorylation of receptor activated Smads (R-Smads), Smad2 and Smad3. R-Smads form a complex with a non-phosphorylated co-Smad, Smad4. The Smad complex is translocated to the nucleus where it recruits transcriptional coactivators like p300 and CBP to induce acetylation and activation of the expression of TGF- β target genes [2].

SnoN and its structurally and functionally related protein Ski are negative regulators of TGF- β -induced transcription. By binding directly to Smads they recruit histone deacetylases (HDAC) to promoter sites and may additionally prevent the nuclear translocation of phosphorylated Smads [3,4]. TGF- β induces rapid degradation of SnoN and Ski to allow the expression of its target genes [5–9]. However, subsequent to the initial repression of SnoN, TGF- β induces the expression of SnoN to reinstate the negative feedback loop [10,11]. Ski and SnoN are implicated as transcriptional corepressors through either interactions or modulation of the activities of hormone receptor corepressors N-CoR/SMRT, mSin3A, MeCP2 and TAF(II)110 [11].

SnoN is widely expressed in adult and embryonic cells. High expression of SnoN is found in many human cancers [11–13]. The level of SnoN is directly linked to its ability to repress TGF- β signaling, but this activity may be cell-type specific [14]. SnoN has been reported to be both a tumor promoter and a tumor suppressor [15]. Over-expression of SnoN leads to resistance to TGF- β -induced growth arrest and formation of mammary tumors in co-operation with polyoma middle T-antigen [16,17]. On the other hand, silencing of SnoN by shRNA expression promotes epithelial-to-mesenchymal transition and metastasis in breast tumorigenesis [18]. We have previously shown that in ER α positive breast cancers, low level of SnoN is a significant prognostic indicator for longer distant disease-free survival [13].

Estrogen receptor α (ER α) is a ligand-inducible nuclear hormone receptor that regulates many physiological processes. Upon ligand binding ER α regulates transcription either by directly binding to DNA on the estrogen-responsive element (ERE) or by interacting with other transcription factors that are already bound to DNA. The regulation of ER α -mediated transcription depends on the formation of multiprotein complexes containing general transcription factors, coactivators, corepressors, cointegrators, histone acetyltransferases, and HDACs [19]. The coactivators frequently bind to activated ER α through leucine rich LxxLL-motifs, so called nuclear receptor binding domain (NBD)-motifs [20,21]. The best characterized coactivators are p160 family member steroid receptor coactivators (SRC-1, SRC-2 and SRC-3) [22,23]. ER α signaling is essential for the development of the mammary gland. Due to its mitogenic activity it is also a driving force during mammary gland tumorigenesis. Approximately 70% of breast cancers are ER α positive and estrogen-dependent. Therefore anti-estrogens like tamoxifen have been the cornerstone in breast cancer therapy for the last 40 years.

Previous reports have demonstrated a cross-talk between ER α and TGF- β signaling pathways [24]. ER α has been shown to physically interact with Smad2, Smad3, and Smad4

and to abrogate TGF- β signaling cascade [25,26]. While TGF- β signaling has been demonstrated to stimulate ER α transcriptional activity, the complex of Smad3/Smad4 inhibits its activity [25,27].

In this study, we demonstrate that the negative regulator of TGF- β signaling pathway, SnoN, interacts with ER α at the ERE promoter and enhances ER α -dependent transcription. This finding defines SnoN as a new ER α coregulator that promotes ER α signaling.

2. Materials and methods

2.1. Cell culture and reagents

293T, COS7, MDA-MB-231, T-47D, U2-OS and HeLa cells were obtained from American Type Culture Collection (Bethesda, MD) and were cultured in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (Autogen Bioclear, Calne, UK). Human breast cancer cell line, MCF7 and MDA-MB-435 melanoma cells were obtained from American Type Culture Collection and were grown in 10% fetal calf serum in Minimum Essential Media (MEM) and in RPMI-1640, respectively. In experiments requiring 17- β -estradiol (E2) free media the cells were cultured in phenol-red free DMEM (Gibco, Invitrogen, Carlsbad, CA) and 4% charcoal/Dextran treated fetal bovine serum (HyClone, Logan, UT). E2 was purchased from Calbiochem (Nottingham, UK), ICI 182,780 and Trichostatin A (TSA) from Tocris Bioscience (Bristol, UK), MG132 from Sigma Aldrich (St. Louis, MO) and Hoechst 33342 from Molecular Probes (Eugene, Oregon).

2.2. Antibodies

Mouse monoclonal antibodies against hemagglutinin (HA)-tag and Myc-tag were from Covance (Princeton, NJ). Mouse monoclonal anti-ER α , anti progesterone receptor (PGR), and rabbit anti-pS2 antibodies were from Novocastra (Newcastle upon Tyne, UK). Rabbit SnoN, Ski, cyclin D1, p300, Bcl-2 and ER α antibodies and mouse monoclonal Sp1, p300 and α -actinin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH) antibody was purchased from Europa Bioproducts (Cambridge, UK). Anti- β -tubulin antibody was from BD Pharmingen (Erembodegen, Belgium). Rabbit Alexa488-conjugated anti-mouse antibody was obtained from Molecular Probes (Eugene, Oregon).

2.3. Plasmids and transient transfection

PCIneoSnoN-HA and PCIneoSki-HA plasmids were kindly provided by Dr. Sunshuke Ishii (RIKEN Tsukuda Institute, Ibaraki, Japan). The negative control siRNA and SnoN-specific siRNAs were purchased from Ambion (Austin, Texas). Cells were transiently transfected with Eugene 6 or Eugene HD reagent (Roche Diagnostics, Mannheim, Germany). 293T cells were transfected with JetPEI (PolyPlus Transfection, Illkirch, France). MCF7 and T-47D cells were transfected with JetPEI or Amaxa (Lonza, Basel, Switzerland).

2.4. Immunofluorescence microscopy

The cells were fixed with 3.5% PFA, permeabilized with 0.5% NP-40 and stained with monoclonal anti-HA antibody. DNA was stained with Hoechst 33342. Images were captured with Zeiss Axioplan 2 MOT epifluorescence microscope, Zeiss Plan-Neofluar objective, Zeiss AxioCam HRm 14-bit grayscale CCD camera and Zeiss Axiovision 4.6 software.

2.5. Cell lysates, immunoblotting and immunoprecipitation

Western blotting and immunoprecipitations were performed as previously reported [28].

2.6. Nuclear and cytosolic fractionation

Cells were washed with PBS and lysed in hypotonic buffer (20 mM Tris-HCl pH 7.4, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 µg/mL leupeptin and 10 µg/mL soybean trypsin inhibitor, as previously reported [28].

2.7. Reporter assay

Dual-luciferase reporter assay was performed according to the manufacturer's instructions (Promega). Renilla luciferase was used as a control plasmid to normalize all transfections. ERE4-reporter was obtained from Dr. Jorma Palvimo (University of Kuopio, Finland).

2.8. Chromatin immunoprecipitation (ChIP)

We performed ChIP assay by modifying previously reported assay [29]. Briefly, MCF7 cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37 °C for 10 min. Next, the cross-linking was stopped with cold 0.125 M glycine in PBS and the nuclear fraction was separated as reported before [28]. 10 µg of salmon sperm DNA was added to the sonicated nuclear fraction and the pre-clearing was performed with protein G-Sepharose beads and diluted with IP buffer. Immunoprecipitation was performed overnight at 4 °C with specific antibodies after which protein-G Sepharose was added for 2 h at 4 °C. Precipitates were washed sequentially with IP buffer, IP buffer with 0.5 M NaCl, LiCl buffer (0.250 M LiCl, 1 mM EDTA, 0.5% Empigen BB, 0.5% sodium deoxycholate, Tris-HCl pH 8.0) and twice with TE buffer (EDTA Tris-HCl). The precipitated chromatin complexes were removed from the beads and the cross-linking was reversed. 1% SDS, 0.3 M NaCl in TE-buffer was added and RNA was digested with 10 µg RNase for 1 h at 37 °C and proteins were digested with 20 µg proteinase K overnight at 65 °C. DNA was purified with phenol/chloroform/isoamylalcohol extraction and precipitated with ethanol. Semi-quantitative or quantitative PCR was performed with previously reported primers [30,31] which were modified for Dynamo TM SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). For Re-ChIP assay the chromatin complex was removed from the beads by incubating beads with 10 mM DTT at 37 °C.

2.9. Proliferation assay

Cell viability was measured using Cell Proliferation Reagent WST-1 according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

2.10. SiRNA silencing

Small interfering RNAs (siRNA) were from Ambion (Austin, Texas). SiRNAs were transfected to MCF7 and T-47D cells using Amaxa Nucleofection System (Lonza, Basel, Switzerland).

2.11. RT-PCR

RNA was isolated by Trizol extraction and the concentrations were measured using Nano-drop. Total RNA was used for reverse transcription with First-strand Super Script kit (Invitrogen). RT products were used to perform qPCR in triplicate with SYBR GREEN I master mix (Atila Biosystem, Mountain View, CA) on ABI PRISM 7900HT (Applied Biosystems, Foster City, CA). Ct was normalized to the Ct of GAPDH. Fold changes were calculated using delta-delta Ct method. The graph represents pooled data from triplicate samples using two different sets of primers. Error bars, SD.

2.12. Statistical analysis

Statistical analysis was performed by Student's two-tailed *t*-test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. SnoN interacts with activated ER α

We have previously demonstrated that SnoN is a prognostic indicator in ER-positive breast cancers [13]. Breast cancer patients whose tumors express a low level of SnoN have a significantly better prognosis than those with a high level of SnoN. However, this was only observed in patients whose tumors were ER α positive. We have now studied the possible mechanism behind this phenomenon. We first investigated a possible interaction between SnoN and ER α . Since Ski is a structurally and functionally homologous protein to SnoN we also included it in these studies. We ectopically expressed ER α and SnoN or Ski and performed co-immunoprecipitation analyses (Fig. 1A). We found that ER α co-precipitated with both SnoN and Ski. When the cells were pretreated with anti-estrogen ICI 182,780 (ICI) before adding 17- β -estradiol (E2), co-precipitation of ER α was no longer detected with either SnoN or Ski. This suggests that only the activated form of ER α interacts with SnoN and Ski. To verify whether the endogenous proteins interact, we co-precipitated SnoN and ER α in MCF7 breast cancer cells. SnoN and ER α co-precipitation was observed in the presence of E2, and was abolished by treatment with ICI 182,780 (Fig. 1B).

Since both Smad3 and ER α interact with SnoN [8,25], we studied the possibility that they would form a complex. We over-expressed SnoN and ER α in the presence of increasing amounts of Smad3 in 293T cells. We detected co-precipitation of SnoN and ER α in the presence of low levels of Smad3 (Fig. 1C). However, increased amounts of Smad3 greatly reduced the interaction between SnoN and ER α (Fig. 1C). This suggests that Smad3 competes with ER α on binding to SnoN.

3.2. SnoN interaction with ER α is dependent on SnoN LxxLL-motif

SnoN contains two NBD-like LxxLL-like motifs, which are fully conserved among several chordate species (Fig. 2A). The second motif is also conserved in human Ski. We mutated two essential leucines in both motifs to alanines to produce SnoN mutants, M1, M2 and a double mutant M1 + M2 (Fig. 2B). Because SnoN has been detected in both cytoplasm and nucleus [4,13], we first determined the localization of wild-type (wt) SnoN and its mutants. We found that although SnoN wt and M1 were predominantly expressed in the nucleus, M2 mutation caused its marked redistribution to the cytoplasm in U2-OS cells (Fig. 2C). We further studied localization of SnoN wt and its mutants by fractionating 293T cells to nuclear and cytoplasmic fractions. The M2-mutant was more highly expressed in the cytoplasmic fractions than in the nuclear fractions (Fig. 2D). We have earlier shown that in human breast cancers, SnoN has heterogeneous expression either in cytoplasmic or nuclear compartments or is present in both [13]. In order to assess whether ER α activity has an effect on the localization of SnoN or its mutants, we co-transfected the SnoN mutants with ER α and treated the cells with E2 (Fig. 2D). There was no change in localization of SnoN or its mutants by estrogen signaling indicating that ER α activity is not a major determinant of SnoN subcellular localization.

SnoN is targeted by proteasomal degradation by E3 ligases (Smurf2, Arkadia and APC/C) mediated by interaction with Smads [6–9,32]. SnoN M2-mutant was predominantly expressed in the cytoplasm whereas wt SnoN and SnoN M1 mutant were present both in the nucleus and in the cytoplasm. In order to investigate whether the apparent relative decrease of nuclear SnoN, especially in the M2 mutant, was due to increased degradation, we

ectopically expressed SnoN and its mutants and treated the cells with MG132 to prevent proteasomal activity (Fig. 2E). In MG132-treated cells SnoN M2-mutant was expressed also in the nuclear fraction similar to wt SnoN, indicating that the M2-mutant is inherently more unstable than wt SnoN in the nucleus in a manner that depends on proteasome activity (Fig. 2E and Supplemental Fig. S1).

Lastly, we addressed whether mutation of the LxxLL-like domains affects the interaction between SnoN and ER α . SnoN and its mutants were expressed in 293T cells, the cells were fractionated, and SnoN was immunoprecipitated from cytoplasmic and nuclear fractions. ER α co-precipitated with wt SnoN only in the nuclear fraction. Furthermore, mutations in the LxxLL-like domains greatly reduced this interaction (Fig. 2F). Mutations of SnoN LxxLL-like domains did not alter the ability of SnoN to interact with either Smad3 or Ski (Supplemental Fig. S2). These findings indicate that SnoN interacts with ER α through NBD-motifs in the nucleus.

3.3. SnoN increases ER α transcriptional activity

SnoN and Ski are negative transcriptional regulators of TGF- β signaling by increasing the formation of a large co-repressor complex [11]. We therefore investigated whether they affect ER α -dependent transcription. We used the well-characterized ERE-reporter assay [33]. SnoN significantly increased the ERE-reporter activity in 293T cells in a dose-dependent manner by up to three-fold (Fig. 3A). We then tested the effect of SnoN on ERE-reporter in the presence of increasing concentrations of ER α . SnoN was able to stimulate the reporter activity more at a low than high level of ER α expression (Fig. 3B). This suggests that SnoN is not an essential factor for the reporter activity but it is a potent enhancer. Enhancement of reporter activity was shown to be E2 dependent, firstly, because depletion of estrogen from the medium greatly reduced the reporter activity (Fig. 3B, C and F), and secondly because SnoN-mediated stimulation of the reporter activity was totally abolished by specific anti-estrogen, ICI 182,780 (Fig. 3C). We also studied the stimulation of ERE-reporter activity in other cell lines. Both SnoN and Ski were able to stimulate the reporter activity in MDA-MB-435 cells (Fig. 3D), whereas in a human cervical cancer cell line, HeLa, only SnoN was able to do so (Fig. 3E). In order to resolve whether SnoN LxxLL-like domains are required for ER α -modulation, we assessed whether the mutations in these domains affect the activation of the ERE4-reporter. We found that stimulation of the reporter activity was greatly reduced by M1 + M2 mutations (Fig. 3F). This suggests that the ability of SnoN to interact with ER α is directly related to its ability to act as co-regulator of ER α activation.

3.4. SnoN binds ER α target promoter in an E2-dependent manner

The stimulation of ERE4-reporter activity by SnoN and interactions between ER α and SnoN suggested that SnoN could be recruited at the ER-dependent promoter sites. We investigated this possibility by using chromatin immunoprecipitation (ChIP) (Fig. 4A). Estrogen depleted MCF7 breast cancer cells were treated with E2, ICI 182,780 and E2 or vehicle. Both anti-ER α and anti-SnoN antibodies immunoprecipitated ERE-containing *TTF1* (pS2) promoter sequence from the soluble chromatin isolated from E2-treated, but not in mock- or ICI 182,780 -treated cells (Fig. 4A, compare *lanes 3, 6 and 9*). We further investigated whether SnoN is present in the complex with ER α on chromatin by using Re-ChIP assay. We first immunoprecipitated soluble chromatin fraction with an anti-ER α antibody and from that precipitate we performed a second immunoprecipitation with an anti-SnoN antibody (Fig. 4B). In this second immunoprecipitation SnoN co-precipitated with ERE promoter sequence on the *TTF1* promoter (Fig. 4B, *lane 5*). These findings suggest that SnoN binds to ER α activated promoter sites through ER α in an E2-dependent manner.

3.5. SnoN promotes recruitment of p300 to ER α

SnoN/Ski has been reported to both inhibit and activate transcription in a cell type specific manner. They may recruit HDACs to Smad binding element (SBE) at the promoter sites in epithelial cells [11] or inhibit the HDAC activity in myogenin promoter in myoblastic cells [34]. Additionally, they may activate transcription by interacting with p300 in neurons [35]. Therefore we tested the effect of a HDAC I and II inhibitor, Trichostatin A (TSA), on SnoN enhanced ERE4-reporter assay (Fig. 5A). TSA-treatment synergistically potentiated SnoN stimulated reporter activity suggesting that HDAC activity co-modulates the SnoN effect. Analysis for the expression of the ectopically expressed proteins indicated that SnoN levels were increased by TSA treatment whereas ER α expression was unaltered (Fig. 5B). This was further confirmed by treating 293 T cells transfected with SnoN with increasing concentrations of TSA. TSA caused a dose-dependent increase in SnoN (Fig. 5C). However, based on the indication that decreased HDAC activity augments the stimulatory effects of SnoN towards ER α (Fig. 5A and B), we hypothesized that SnoN could act by attracting histone acetylases, like p300 to the ER α complex. ER α was expressed in 293T cells in the presence or absence of SnoN followed by precipitation for p300 bound complexes. SnoN increased the interaction between ER α and p300 (Fig. 5D), suggesting that SnoN may recruit p300 to the ER α transcriptional complex.

3.6. SnoN stimulates the expression of ER-dependent transcription

We then studied the effect of SnoN on ER α signaling in MCF7 cells. MCF7 cells have high endogenous ER α activity in ERE-reporter assays, but the activity was significantly increased by the expression of SnoN and Ski (Fig. 6A). Further, the increase in reporter activity was E2-dependent since treatment with ICI 182,780 totally abolished it (Fig. 6B). To verify that the observed increases in ERE-reporter activity and SnoN chromatin binding impact also ER α target gene expression, we tested the effect of over-expression of either SnoN or Ski in MCF7 cells. Based on immunoblotting for pS2, cyclin D1, Bcl-2 and progesterone receptor (PGR) both SnoN and Ski increased their expression (Fig. 6C). These findings support the conclusion that SnoN regulates transcription of endogenous ER α regulated genes. Consequently, we tested the effect of SnoN on the proliferation of MCF7 cells. MCF7 cells were transfected with SnoN and incubated in the presence of E2 and in the presence or absence of ICI 182,780. We found that although SnoN enhanced both E2-dependent and -independent proliferation, the enhancement of E2-dependent proliferation was much more marked (Fig. 6D).

3.7. SnoN silencing represses ER-dependent transcription and gene expression

Conversely, we tested the effect of silencing of endogenous SnoN on ER-dependent transcription in MCF7 cells. SnoN was effectively silenced using siRNA transfection, and this led to significant reduction of ERE4 reporter activity (Fig. 7A). The degree of reduction of ER α activity following SnoN silencing was dependent on the supplied E2 concentration, i.e. degree of ER α stimulus (Supplemental Fig. S3) supporting the notion that silencing of SnoN has a greater impact on the transcriptional activity of ER α at below maximal ER α activity. Silencing of SnoN also reduced the expression of ER α target genes like *TTF1* and *PGR* at both mRNA and protein level (Fig. 7B and C). The above results, using both SnoN over-expression and silencing, were confirmed using another ER α positive cell breast cancer cell line, T-47D. Over-expression of SnoN and Ski in T-47D potentiated ERE-reporter assay activity and increased PGR protein expression (Fig. 8A and B). Additionally, silencing of SnoN reduced both mRNA and protein levels of PGR in T-47D cells (Fig. 8C and D).

4. Discussion

In this study we show that SnoN is a potent coactivator of ER α signaling. We find that SnoN interacts with ER α in a manner dependent on activated, nuclear ER α . SnoN contains two well-conserved LxxLL-like motifs that are common motifs among nuclear receptor binding proteins [20,21]. These motifs are necessary to mediate the binding of the coactivator to the activated ER α . Estrogen binding to the ligand-binding domain (LBD) of ER α leads to ER α conformational change, receptor dimerization, nuclear transport and binding of its coactivators. Coactivators typically bind to the ER α hydrophobic groove that serves as a pocket for the NBD-motif containing proteins. When we mutated double leucines to alanines in the LxxLL-like motifs (M1 and M2), the mutation of especially the M2 site reduced SnoN interaction with ER α and its coactivator activity. Interestingly, the second LxxLL-like motif is fully conserved in Ski. In contrast to the classical LxxLL motif, the SnoN LxxLL-like domain contains three small amino acids located between the leucines. Recent publication of the crystal structure of SnoN N-terminal Dachshund homology domain (DHD), which contains the M2 LxxLL-like domain, indicates that the DHD domain has the characteristic properties of a protein-interaction surface [36]. SnoN is expressed predominantly in cytoplasm, but also in the nucleus of cancer cell lines, melanomas and in breast cancer [11,13]. Using subcellular fractionation we show here that mutated SnoN is unstable in the nuclear compartment in a manner that depends on proteasome activity. This is suggestive that SnoN interaction with ER α or with other proteins protects it from degradation in the nucleus.

The downstream signaling components of TGF- β , Smad2, Smad3 and Smad4, have been previously reported to bind ER α and to regulate ER α signaling [25–27]. TGF- β signaling is reported to stimulate ER α activity and the complex of Smad3/4 to inhibit it [25,27]. Conversely, ER α inhibits TGF- β signaling by decreasing Smad3 levels [26], indicating an intricate cross-regulation by the two major signaling pathways. On the other hand, SnoN binds Smads with high affinity and the binding of Smad2 or Smad3 recruits ubiquitin ligase to the complex and leads to ubiquitylation and degradation of SnoN by the proteasome [6,7,37]. Therefore we tested whether the interaction of SnoN and ER α requires Smad3. We over-expressed SnoN, ER α , and an increasing amount of Smad3. Over-expression of Smad3 led to dissociation of the SnoN and ER α complex. These results suggest that Smad3 may abrogate the complex formation between SnoN and ER α . Ski was included in some experiments since Ski is structurally and functionally homologous to SnoN [11]. It seems that Ski is also able to bind and stimulate ER α -induced transcription, which may be relevant in the context of cells that highly express Ski.

We show here that SnoN is a novel coactivator of ER α . ERE reporter assays showed that SnoN is a potent stimulator of ER α -dependent transcription. SnoN was able to stimulate the ERE-reporter activity by two- to three-fold in all cell lines tested, and this activity was quenched by both depletion of the ligand and by the pure ER antagonist ICI 182,780. Sequential ChIP assays revealed that SnoN interacts with ER α at the *TTF1* promoter in MCF7 cells. Furthermore, over-expression of SnoN led to an increase in ER α targets in MCF7 and T-47D cells. Conversely, silencing of SnoN reduced their expression. These results indicate that SnoN interacts with the activated ER α bound at chromatin. The *TTF1* gene has a classical ERE promoter where ER α directly binds to DNA and recruits basal transcription factors and coregulators to regulate the transcription of target genes [19]. ER α also regulates gene transcription through binding to the other transcription factors such as Sp1, AP1, and Fox-A1 [19,38,39]. ER α regulates the transcription of cyclin D1, anti-apoptotic protein Bcl-2 and PGR through these nonclassical sites. Given that overexpression of SnoN potentiated their expression it is likely that SnoN is involved in the regulation of ER α dependent transcription at these non-classical promoters as well. Further, the binding

of p300 to ER α in the presence of SnoN is suggestive that increased acetylation of ER α , or other complex members, is at least one modality how SnoN promotes ER α activity.

SnoN regulates mouse mammary gland morphogenesis and undergoes alterations in breast cancer. MMTV-driven SnoN expression led to lobular-alveolar hyperproliferation and increased branching morphogenesis of the breast, whereas mammary glands of SnoN knockout mice were hypoplastic [17]. Further, MMTV-driven SnoN promoted tumorigenesis with polyoma virus middle T-antigen, which increased lung metastasis and decreased survival [17]. We have previously demonstrated that the level of SnoN correlates with tumor progression in human breast cancer [13]. We found that a low SnoN level was associated with longer disease-free survival when assessed with immunohistochemical analysis in a tissue microarray of over 1000 breast carcinomas. The difference was particularly striking among patients with ER α positive tumors. The mechanism behind this phenomenon has been unclear. Hence, the current study provides a mechanistic explanation how SnoN influences ER α signaling, and demonstrates that SnoN is a potent coactivator of ER α . The present findings are consistent with the interpretation that high SnoN levels may promote ER α signaling and breast cancer progression.

5. Conclusion

Our findings demonstrate that the negative regulator of TGF- β signaling, SnoN, acts as a coactivator in the ER α signaling cascade. This study reveals a novel mechanism that interconnects ER α and TGF- β signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Päivi Heikkilä and Laiholab members for discussion, Hester Liu, Maija Salo, Anni-Helena Sukupolvi, Kaisa Penttilä, and Runar Ra for excellent technical assistance. We thank Dr. Aris Moustakas, Dr. Carl-Henrik Heldin, Dr. Sunshuke Ishii and Dr. Jorma Palvimo for expression vectors. This work was supported by Academy of Finland (grant no. 129699).

Abbreviations

ChIP	chromatin immunoprecipitation
ER	estrogen receptor
ERE	estrogen response element
E2	17- β -estradiol
HA	hemagglutinin
HDAC	histone deacetylases
NBD	nuclear receptor binding domain
SnoN	ski-related novel gene
TGF-β	transforming growth factor- β
TSA	trichostatin A

References

1. Massagué J. *Cell*. 2008; 134:215–230. [PubMed: 18662538]
2. Ross S, Hill CS. *The International Journal of Biochemistry & Cell Biology*. 2007; 40:383–408.
3. Luo K. *Current Opinion in Genetics and Development*. 2004; 14:65–70. [PubMed: 15108807]
4. Krakowski AR, Laboureaux J, Mauviel A, Bissell MJ, Luo K. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:12437–12442. [PubMed: 16109768]
5. Sun Y, Liu X, Ng-Eaton E, Lodish HF, Weinberg RA. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:12442–12447. [PubMed: 10535941]
6. Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL. *Nature Cell Biology*. 2001; 3:587–595.
7. Stroschein SL, Bonni S, Wrana JL, Luo K. *Genes & Development*. 2001; 15:2822–2836. [PubMed: 11691834]
8. Wan Y, Liu X, Kirschner MW. *Molecular Cell*. 2001; 8:1027–1030. [PubMed: 11741538]
9. Levy L, Howell M, Das D, Harkin S, Episkopou V, Hill CS. *Molecular and Cellular Biology*. 2007; 27:6068–6083. [PubMed: 17591695]
10. Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. *Science*. 1999; 286:771–774. [PubMed: 10531062]
11. Deheuninck J, Luo K. *Cell Research*. 2009; 19:47–57. [PubMed: 19114989]
12. Imoto I, Pimkhaokham A, Fukuda Y, Yang ZQ, Shimada Y, Nomura N, Hirai H, Imamura M, Inazawa J. *Biochemical and Biophysical Research Communications*. 2001; 286:559–565. [PubMed: 11511096]
13. Zhang F, Lundin M, Ristimäki A, Heikkilä P, Lundin J, Isola J, Joensuu H, Laiho M. *Cancer Research*. 2003; 63:5005–5010. [PubMed: 12941827]
14. Sarker KP, Wilson SM, Bonni S. *Journal of Biological Chemistry*. 2005; 280:13037–13046. [PubMed: 15677458]
15. Lamouille S, Derynck R. *EMBO Journal*. 2009; 28:3459–3460. [PubMed: 19920850]
16. Edmiston JS, Yeudall WA, Chung TD, Lebman DA. *Cancer Research*. 2005; 65:4782–4788. [PubMed: 15930298]
17. Jahchan NS, You YH, Muller WJ, Luo K. *Cancer Research*. 2010; 70:4204–4213. [PubMed: 20460516]
18. Zhu Q, Krakowski AR, Dunham EE, Wang L, Bandyopadhyay A, Berdeaux R, Martin GS, Sun L, Luo K. *Molecular and Cellular Biology*. 2007; 27:324–339. [PubMed: 17074815]
19. Cheskis BJ, Greger JG, Nagpal S, Freedman LP. *Journal of Cellular Physiology*. 2007; 213:610–617. [PubMed: 17886255]
20. Heery DM, Kalkhoven E, Hoare S, Parker MG, Heery DM, Kalkhoven E, Hoare S, Parker MG. *Nature*. 1997; 387:733–736. [PubMed: 9192902]
21. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG. *Nature*. 1997; 387:677–684. [PubMed: 9192892]
22. Edwards DP. *Journal of Mammary Gland Biology and Neoplasia*. 2000; 5:307–324. [PubMed: 14973393]
23. Schiff R, Osborne CK. *Breast Cancer Research*. 2005; 7:205–211. [PubMed: 16168139]
24. Band AM, Laiho M. *Journal of Mammary Gland Biology and Neoplasia*. 2011; 16:109–115. [PubMed: 21390570]
25. Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F. *Journal of Biological Chemistry*. 2001; 276:42908–42914. [PubMed: 11555647]
26. Ito I, Hanyu A, Wayama M, Goto N, Katsuno Y, Kawasaki S, Nakajima Y, Kajiro M, Komatsu Y, Fujimura A, Hirota R, Murayama A, Kimura K, Imamura T, Yanagisawa J. *Journal of Biological Chemistry*. 2010; 285:14747–14755. [PubMed: 20207742]
27. Ren Y, Wu L, Frost AR, Grizzle W, Cao X, Wan M. *Molecular Cancer*. 2009; 8:111. [PubMed: 19943940]

28. Band AM, Björklund M, Laiho M. *Journal of Biological Chemistry*. 2009; 284:35441–35449. [PubMed: 19875456]
29. Tuupanen S, Turunen M, Lehtonen R, Hallikas O, Vanharanta S, Kivioja T, Björklund M, Wei G, Yan J, Niittymäki I, Mecklin JP, Järvinen H, Ristimäki A, Di-Bernardo M, East P, Carvajal-Carmona L, Houlston RS, Tomlinson I, Palin K, Ukkonen E, Karhu A, Taipale J, Aaltonen LA. *Nature Genetics*. 2009; 41:885–890. [PubMed: 19561604]
30. Wang LH, Yang XY, Zhang X, An P, Kim H-J, Huang J, Clarke R, Osborne CK, Inman JK, Appella E, Farrar WL. *Cancer Cell*. 2006; 10:487–499. [PubMed: 17157789]
31. Chatagnon A, Ballestar E, Esteller M, Dante R. *PLoS One*. 2010; 5:e9665. [PubMed: 20300195]
32. Itoh S, ten Dijke P. *Current Opinion in Cell Biology*. 2007; 19:176–184. [PubMed: 17317136]
33. Klinge CM. *Nucleic Acids Research*. 2001; 29:2905–2919. [PubMed: 11452016]
34. Kobayashi N, Goto K, Horiguchi K, Nagata M, Kawata M, Miyazawa K, Saitoh M, Miyazono K. *Genes to Cells*. 2007; 12:375–385. [PubMed: 17352741]
35. Ikeuchi Y, Stegmüller J, Netherton S, Huynh MA, Masu M, Frank D, Bonni S, Bonni A. *Journal of Neuroscience*. 2009; 29:4312–4321. [PubMed: 19339625]
36. Nyman T, Trésaugues L, Welin M, Lehtiö L, Flodin S, Persson C, Johansson I, Hammarström M, Nordlund P. *PLoS One*. 2010; 5:e12907. [PubMed: 20957027]
37. Band, A.; Laiho, M. Transforming Growth Factor- β in Cancer Therapy. Jakowlew, S., editor. New York: Humana Press; 2008. p. 243-257.
38. Green KA, Carroll JS. *Nature Reviews. Cancer*. 2007; 7:713–722.
39. Safe S, Kim K. *Journal of Molecular Endocrinology*. 2008; 41:263–275. [PubMed: 18772268]

Fig. 1. SnoN and ERα interact in vitro and in vivo

(A) COS7 cells were transiently transfected with ERα, SnoN-HA and Ski-HA expression vectors as indicated. The cells were treated with ICI 182,780 (ICI, 1 μM) as indicated for 4 h prior to addition of E2 (100 nM) for an hour. The cells were then lysed, and the lysates were immunoprecipitated (IP) with anti-HA antibody followed by immunoblotting (WB) with anti-ERα and HRP-conjugated anti-mouse antibody and then incubated with anti-HA antibody followed by HRP-conjugated anti-mouse antibody. Input lysates are shown below.

(B) MCF7 cells were grown in charcoal-treated medium overnight and were then treated with ICI (1 μM) for 4 h before addition of E2 (10 nM) for an hour as indicated. The cells were lysed, immunoprecipitated with control IgG or anti-SnoN antibody and immunoblotted with anti-SnoN or anti-ERα antibodies. Input lysates are shown below.

(C) Smad3 competes with ERα for the binding of SnoN. 293T cells were co-transfected with SnoN-HA and ERα and with an increasing amount of Smad3-Myc expression vector. Nuclear (N) and cytosolic (C) fractions were prepared. SnoN was immunoprecipitated and immunoblotting was performed with anti-HA, anti-ERα, and anti-Myc antibodies. β-Tubulin and Sp1 were used as cytosolic and nuclear markers, respectively.

Fig. 2. SnoN interacts with ERα in an NBD-domain dependent manner

(A) Conservation of the putative SnoN and Ski NBD-motifs across species. (B) SnoN domains and generation of SnoN NBD-mutants. (C) U2-OS cells were transfected with wild-type (wt) SnoN or SnoN mutants as indicated, and immunostained using anti-HA antibody followed by Alexa488-conjugated anti-mouse antibody and DNA was visualized with Hoechst staining. Bars, 10 μm. (D) 293T cells were transiently transfected with wild-type (wt) SnoN or SnoN mutants and ERα, as indicated. After subcellular fractionation lysates were blotted with anti-HA, anti-α-actinin, anti-Sp1 and anti-ERα antibodies. α-Actinin and Sp1 were used as cytosolic (C) and nuclear (N) markers, respectively. (E) 293T cells were transiently transfected with wt SnoN or its mutants. The cells were treated with MG132 (15 μM) for 17 h as indicated. Cytosolic and nuclear fractions were separated and lysates were immunoblotted with anti-HA, anti-Sp1 and anti-β-tubulin antibodies. β-Tubulin and Sp1 were used as cytosolic and nuclear markers, respectively. (F) 293T cells were transfected with wt SnoN-HA and SnoN-HA mutants M1, M2 and M1 + M2 and ERα. The cells were treated with E2 (10 nM) for an hour and then separated into cytosolic (C) and nuclear (N) fractions. The lysates were immunoprecipitated with anti-HA antibody and probed with anti-HA and anti-ERα antibodies. α-Actinin and Sp1 were used as cytosolic

and nuclear markers, respectively. The data shown are representative of three similar experiments.

Fig. 3. SnoN enhances ERα-dependent transcriptional activity

(A) 293T cells were transfected with ERE4-Luc together with ERα and an increasing concentration of SnoN. After transfection the cells were grown in the presence of E2 (100 nM) for 24 h after which the reporter activity was determined based on firefly luciferase activity. All samples were normalized against *Renilla* luciferase used as a transfection control. Western blotting was used to control the expression of the respective transfected proteins. (B) 293T cells were grown in charcoal-treated medium and transfected with ERE4-Luc, SnoN and increasing amounts of ERα and were treated with E2 (10 nM) or vehicle for 24 h as indicated. (C) 293T cells were grown in charcoal-treated medium, transfected as above and treated with ICI 182,780 (1 μM) and E2 (10 nM) or vehicle for 24 h as indicated. (D) MDA-MB-435 cells were grown in charcoal-treated medium and were transfected with ERE4-Luc reporter, ERα, SnoN or Ski. The cells were treated with E2 for 24 h. (E) HeLa cells were treated as in D. (F) 293T cells were grown in charcoal-treated medium and transfected with ERE4-Luc, ERα and either with wt SnoN or SnoN M1 + M2 mutant and treated with E2 or vehicle for 24 h as indicated. All data represents average of at least three replicates and is representative of three similar experiments. Error bars, SD. *P*-values are indicated by asterisks, * ($P < 0.05$), ** ($P < 0.01$), ns; non-significant.

Fig. 4. SnoN interacts with ERα at the *TTF1* promoter

(A) MCF7 cells were grown in charcoal-treated medium, treated with ICI 182,780 (ICI, 1 μ M) or DMSO for 24 h, and E2 or vehicle (veh) was added for an hour. Chromatin immunoprecipitation (ChIP) was performed with control IgG, anti-ERα and anti-SnoN antibodies. PCR was performed using primers specific for *TTF1* promoter. Input represents 25% of total input. (B) MCF7 cells were grown in normal medium and E2 was added for an hour before isolating the chromatin for the first ChIP assay (ChIP: 1). The ERα precipitates were subjected to a second ChIP (ChIP: 2) using anti-SnoN antibody or control IgG antibody. Three times more input was used for ChIP: 2. Representative experiments are shown.

Fig. 5. SnoN promotes the association between p300 and ERα

(A) 293T cells were grown in charcoal-treated medium, transfected with ERE4-Luc, ERα, SnoN and Ski and treated with vehicle, E2 and TSA (1 μM) as indicated for 24 h and reporter activity was determined. (B) Western blotting of the reporter assay A. The cell lysates were immunoblotted with anti-HA, anti-ERα and anti-α-actinin antibodies. (C) 293T cells transfected with SnoN expression vector were treated with increasing concentrations of TSA for 24 h, lysed and immunoblotted with anti-HA and anti-α-actinin antibodies. (D) (F) 293T cells were co-transfected with ERα and SnoN-HA as indicated. Endogenous p300 was immunoprecipitated and immunoblotting was performed with anti-p300, anti-ERα, and anti-HA antibodies. Representative experiments are shown. Error bars, SD. *P*-values are indicated by asterisks, **(*P*<0.01), *** (*P*<0.001).

Fig. 6. SnoN and Ski stimulate ER α activity in MCF7 cells

(A) MCF7 cells were transfected with ERE4-Luc reporter and SnoN or Ski, as indicated. The cells were grown in charcoal-treated medium in the presence of E2 (100 nM). Data represents average of three independent experiments. Error bars, SE. (B) MCF7 cells were transfected with ERE4-Luc reporter and SnoN and treated with ICI 182,780 (1 μ M) and E2 (10 nM) for 24 h, as indicated. The reporter activity was determined. Data represents average of three replicates. (C) MCF7 cells were transfected with SnoN or Ski and maintained in charcoal-treated medium in the presence of E2 for 20 h. Cell lysates were immunoblotted with anti-HA, anti-pS2, anti-cyclin D1, anti-Bcl-2, anti-progesterone receptor (PGR), and anti- β -tubulin antibodies. (D) MCF7 cells were transfected with SnoN

and treated with ICI 182,780 (1 μ M) and E2 (10 nM) for 5 days, as indicated. The proliferation of the cells was measured with WST-1 assay. The data was quantified from three independent experiments. Error bars, SD. *P*-values are indicated by asterisks, **(*P*<0.01), *** (*P*<0.001).

Fig. 7. Silencing of SnoN abrogates ER α activity in MCF7 cells

(A) MCF7 cells were transfected either with control siRNA or SnoN specific siRNA and incubated for 40 h. Cells were then transfected with ERE4-Luc reporter and treated with ICI 182,780 (1 μ M) or vehicle (veh) for 24 h, as indicated. Data represents average of two independent experiments each performed in triplicate. (B) Western blotting of cell lysates transfected with control siRNA or SnoN specific siRNA as in A. Cell lysates were immunoblotted with anti-SnoN, anti-pS2, anti-cyclin D1, anti-Bcl-2, anti-progesterone receptor (PGR), and anti- α -tubulin antibodies. (C) MCF7 cells were grown in charcoal-treated medium in the presence of E2 (1 nM) for 20 h. Total RNA was isolated and *TTF1* (pS2) mRNA and *PGR* mRNA were analyzed using quantitative RT-PCR. Error bars, SD. *P*-values are indicated by asterisks, **(*P*<0.01).

Fig. 8. SnoN regulates ERα activity in T-47D cells

(A) The cells were grown in charcoal-treated medium, transfected with ERE4-Luc reporter, SnoN and Ski, treated with ICI 182,780 (1 μM) and E2 (10 nM) for 24 h, as indicated. The reporter activity was determined. Data represents average of two independent experiments. Error bars, SD. (B) The cells were transfected with SnoN or Ski and were grown in charcoal-treated medium in the presence of E2 (10 nM) for 20 h. Cell lysates were immunoblotted with anti-HA, anti-progesterone receptor (PGR), and anti-α-tubulin antibodies. (C) The cells were transfected either with control siRNA or SnoN specific siRNA and incubated for 48 h, lysed and immunoblotted with anti-SnoN, anti-progesterone receptor (PGR), and anti-α-tubulin antibodies. (D) Cells were transfected with control

siRNA or SnoN siRNA and incubated for 40 h. The medium was then replaced with charcoal-treated medium, E2 (1 nM) was added and the cells were incubated for further 16 h. Total RNA was isolated and *PGR* mRNA was analyzed using qRT-PCR. Error bars, SD. *P*-values are indicated by asterisks, * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$).