UNIVERSITY OF CALGARY

Identification and Functional Characterization of SnoN Sumoylation

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

AUGUST, 2006

UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled " Identification and Functional Characterization of SnoN Sumoylation" submitted by Ying-Han Hsu in partial fulfilment of the requirements of the degree of Master of Science.

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Abstract

The transcriptional modulator SnoN controls a diverse set of biological processes including cell proliferation and differentiation. However, the mechanisms by which SnoN regulates these processes remain incompletely understood. Recent studies have shown that SnoN exerts positive or negative regulatory effects on transcription. Since posttranslational modification of proteins by SUMO represents an important mechanism in the control of the activity of transcriptional regulators, the work in this thesis project examined if this modification regulates SnoN function. This study shows that SnoN is sumoylated. The data demonstrate that the SUMO-conjugating E2 enzyme Ubc9 is critical for SnoN sumoylation and that the SUMO E3 ligase PIAS1 selectively interacts with and enhances the sumoylation of SnoN. Lysine residues 50 and 383 are identified as the SUMO acceptor sites in SnoN. Analyses of SUMO "loss of function" and "gain of function" SnoN mutants in transcriptional reporter assays reveal that sumoylation of SnoN contributes to the ability of SnoN to repress gene expression in a promoter-specific manner. While this modification has little effect on SnoN-repression of PAI-1 promoter and modestly potentiates SnoN-repression of the p21-gene promoter, SnoN sumoylation robustly augments the ability of SnoN to suppress transcription of the myogenesis master regulatory gene myogenin. Together, the results of this study suggest that SnoN is directly regulated by sumoylation leading to the enhancement of SnoN's ability to repress transcription in a promoter-specific manner.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Shirin Bonni for her tremendous support and guidance and for giving me the opportunity to work on this interesting project. I would also like to thank my supervisory committee members, Drs. Don Fujita and Frank Jirik for their helpful advice during the course of this project.

In addition, during my two years at the Southern Alberta Cancer Research Institute, I have been blessed with wonderful friends to whom I will always be grateful. I would like to say a big "Thank You" to Dr. Krishna Sarker for being a mentor and a great friend who is instrumental in this project; to Sylvia and Jocelyn for showing me the laboratory techniques; to Jeff, Xiaolan and King for their friendship and laughter; to Mary and Melanie for their cheerful support and friendship; to Feng for her wonderful assistance; to Kendel, Amber and Angela for their tremendous help in the lab; to Dr. Isabelle Pot for her collaboration and helpful discussions in this project; and to Dr. Stuart Netherton for his helpful advice and discussions.

Finally, I would like to express my thanks to Drs Tara Beattie, Chris Brown, Peter Forsyth, Randal Johnston, Justin MacDonald, Derrick Rancourt and Stephen Robbins and their friendly lab members for their generosity and kind assistance in the sharing and operation of equipment.

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List of Symbols, Abbreviations and Nomenclature

Abbreviations	Definition
BSA	Bovine serum albumin
dNTPs	Deoxynucleotides
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HDAC	Histone deacetylase
IB	Immunoblotting
IF	Immunofluorescence
IP	Immunoprecipitation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOEing	Splicing by Overlap Extension
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered saline plus 0.1% Tween-20
TGF-β	Transforming growth factor-beta
WB	Western blotting

Chapter One: Introduction

1.1 Overview

The human *sno* (ski-related novel) gene was originally discovered as a close homologue of c-*ski*, the cellular counterpart of the Sloan-Kettering avian transforming viral gene, v-*ski* (Nomura et al., 1989); together, these genes form the founding members of the *ski*/*sno* family of proto-oncogenes. *sno* is also distantly related to *dach*, the mammalian homologue of the *Drosophila* retina determination gene, *dachshund* (Hammond et al., 1998; Kozmik et al., 1999). Proteins encoded by the *sno*/*ski*/*dach* gene family share a 100 amino acid homology region at their amino termini (Figure 1-1), and are believed to have transcriptional regulatory functions and may control various signalling pathways including the bone morphogenic protein (BMP), transforming growth factor (TGF)-β and Wnt signalling pathways (Wu et al., 2003; Luo, 2004; Reed et al., 2005). Consequently, the Sno/Ski/Dach family of proteins impact important cellular processes including myogenesis, neurulation, eye formation, and morphological transformation (Liu et al., 2001; Luo, 2004; Hanson, 2001). For instance, SnoN, an alternatively spliced product of the *sno* gene (see Section 1.2), has been shown to play a role in oncogenic transformation, in part by antagonizing the TGF-β-induced growth inhibition (Liu et al., 2001; Luo, 2004). Thus, in recent years, there has been much interest in understanding the mechanisms by which SnoN and its related members affect these important cellular processes. This thesis project focuses on SnoN and aims to explore sumoylation of SnoN as one possible mechanism to regulate SnoN functions.

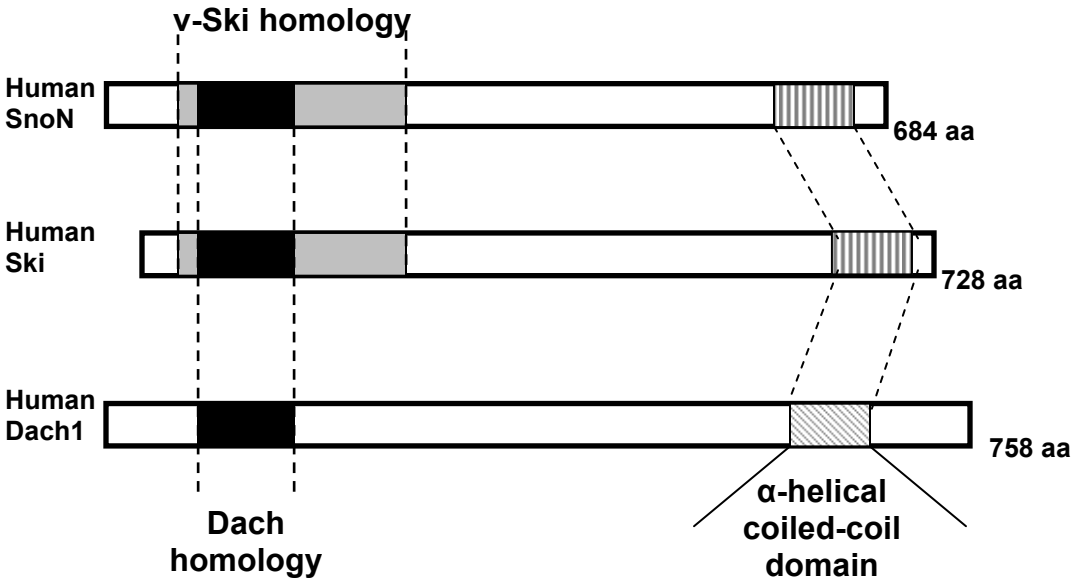


Figure 1-1 Schematic representation showing similarities among SnoN, Ski and Dach1

SnoN and Ski share with Dach1 the Dach homology region of 100 amino acids within the v-Ski homology region. SnoN and Ski share high degree of structural similarity in the C-terminal coiled-coil domain, while Dach1 shows only weak similarity in that region (Hammond et al., 1998).

1.2 The *sno* gene products

Sno protein is expressed in diverse vertebrate species including human, mouse, chicken and frog, and its sequence is highly conserved across species (Pearson-White, 1993). *In situ* hybridization studies showed that the *sno* gene product is expressed during embryonic as well as adult stages (Pelzer et al., 1996; Pearson-White and Crittenden, 1997). Sno mRNA expression is observed in many tissues including lung, kidney, liver, brain and muscle (Nomura et al., 1989; Pearson-White, 1993; Pearson-White and Crittenden, 1997)

In humans, four alternative splice variants of Sno have been reported, designated SnoN, SnoN2, SnoA and SnoI (Nomura et al., 1989; Pearson-White, 1993; Pearson-White and Crittenden, 1997) as shown in Figure 1-2. All four isoforms share a 366-amino acid region derived from exon 1. SnoA or Sno that contains *Alu*I-like restriction site consensus sequence has a unique region resulting from the use of an alternative exon 2. The longest form of the *sno* gene product, SnoN (Non-*Alu* containing) lacks this sequence as a result of the use of a mutually exclusive exon 2 (Nomura et al., 1989). SnoN2 results from an exclusion of 138 nucleotides in exon 3 by an alternative 5' splice donor site (Pelzer et al., 1996; Pearson-White and Crittenden, 1997). SnoI (Insertion) is produced by alternatively spliced exon 3 resulting in a truncated form of SnoN (Pearson-White, 1993).

SnoN is found in all vertebrate species tested thus far and expressed in many tissues (Pearson-White, 1993; Boyer et al., 1993). In chicken, SnoN is the only form produced. Rodents only express SnoN and SnoN2 with varying tissue distributions (Pelzer et al., 1996; Pearson-White and Crittenden, 1997). In the brain, they are

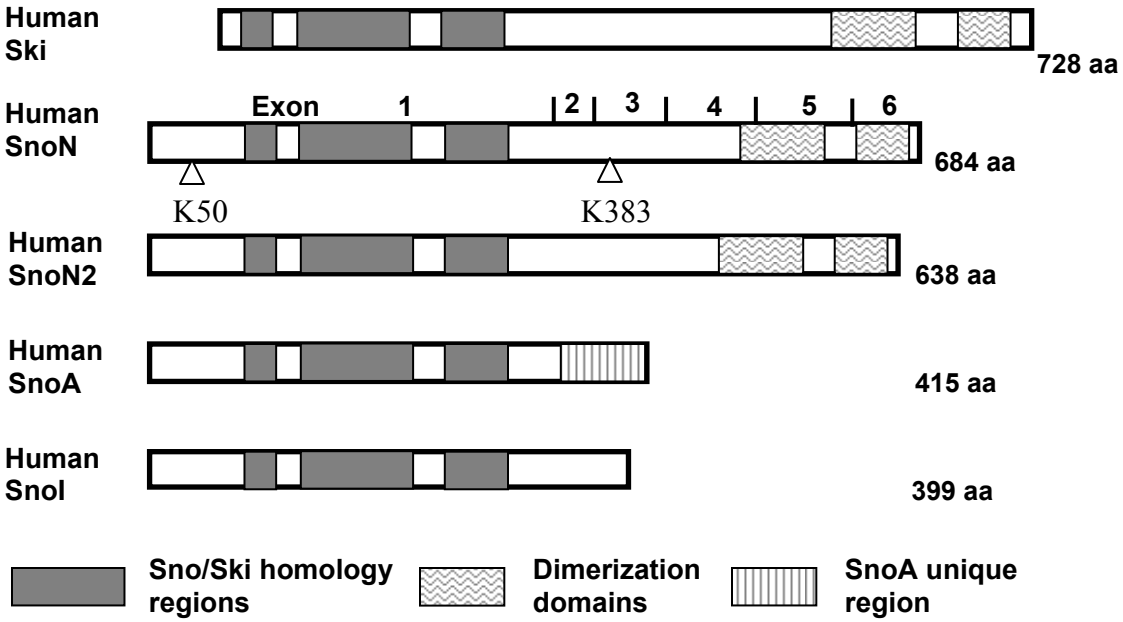


Figure 1-2 Schematic representation of Sno isoforms and Ski showing regions of homology and divergence

All of the Sno splice variants share a common exon 1, which contains a region homologous to Ski. SnoN2 is produced by a deletion of 138 nucleotides in exon 3 as a result of using a different 5' splice donor. SnoN and SnoN2 contain the C-terminal dimerization domains that share similarity with Ski. SnoA is produced by the use of an alternative exon 2. SnoI is a truncated form of SnoN, resulting from a nucleotide insertion in an alternative exon 3 that codes for a stop codon. The relative positions of the putative lysine residues for sumoylation (see Figure 1-5) with respect to the exon structure are indicated in SnoN. (adapted from Pearson-White and Crittenden, 1997)

expressed equally; whereas, in liver and small intestine, SnoN2 is more abundant than SnoN. In humans, SnoN and SnoA are expressed in many tissues with SnoN being the more abundant form (Nomura et al., 1989; Pearson-White, 1993). SnoN2 is much less abundantly expressed and SnoI appears to accumulate only in skeletal muscle (Pearson-White, 1993; Pearson-White and Crittenden, 1997). SnoN and SnoN2 mRNAs are both upregulated by serum stimulation of quiescent mouse fibroblasts, with SnoN2 showing a more robust increase (Pearson-White and Crittenden, 1997). The functional implication of the various Sno splice forms is currently unknown.

1.3 SnoN functional domains

Initial studies showed that a high level of SnoN can transform chicken embryonic fibroblasts (Boyer et al., 1993). Later, studies were done to understand the mechanism by which SnoN transforms cells. Domain-mapping studies using deletion mutants of SnoN pointed to several important conserved regions that offered some mechanistic details for the transforming activity of SnoN (Heyman and Stavnezer, 1994; Cohen et al., 1998; Cohen et al., 1999). The N-terminal half (~260 amino acids) of the Ski/SnoN family shares a high degree of homology. This conserved region of SnoN is overall 60% identical to Ski, and is both necessary and sufficient for oncogenic transformation of avian fibroblasts in both SnoN and Ski (Zheng et al., 1997; Cohen et al., 1998). The C-terminal region of SnoN contains two α-helical coiled-coil domains consisting of a heptad tandem repeat of 25 amino acids and a leucine-zipper-like motif that mediate homodimerization and heterodimerization with c-Ski (Nagase et al., 1993; Heyman and Stavnezer, 1994). Heterodimers of SnoN and Ski exhibit an enhanced transforming ability as compared to monomers or homodimers (Cohen et al., 1999).

Studies in the early 1990's indicated that SnoN binds specific DNA elements *in vitro* through an unknown cofactor, pointing to the possibility that SnoN may be a transcription regulator (Nagase et al., 1993). Further work demonstrated that SnoN has transcriptional repressive activity. Cohen et al (1998) mapped repression domains of SnoN that associated with a GTCTAGAC DNA element. Two subdomains responsible for SnoN-mediated cellular transformation and transcription repression were located within the amino terminal Sno/Ski homology region. A third subdomain at the amino terminal unique region of SnoN appeared to further enhance repression (Figure 1-2).

A decade after its discovery, several groups independently demonstrated that SnoN interacts with the TGF-β receptor regulated Smads, Smad2 and Smad3 as well as the tumor suppressor, Smad4 (Stroschein et al., 1999; Sun et al., 1999a; Akiyoshi et al., 1999; Luo et al., 1999; Xu et al., 2000). Consistently, the GTCTAGAC DNA sequence which SnoN was found to indirectly bind turned out to be a consensus Smad Binding Element (SBE). Recent crystallographic data of a complex between the Smad4-binding region of Ski and the MH2 (Mad homology domain 2) of Smad4 provided insightful information on the structural and functional significance of SnoN and Ski (Wu et al., 2002). The highly conserved N-terminal region of Ski (and SnoN) contains a Cys-His motif that coordinates a zinc atom and is required for structural stability. Located C-terminal to the zinc-binding motif is the interaction surface of Ski with Smad4, which displays similarity to the SAND domain (Sp100, AIRE-1, NucP41/75 and DEAF-1) shared by several chromatin-binding transcriptional factors. These data are consistent with earlier observations that deletion of either the zinc-binding motif or the SAND

domain completely abolishes the transforming capabilities of the Ski/SnoN family proteins (Zheng et al., 1997).

Most studies have supported the idea that SnoN is a nuclear protein. However, recent studies have demonstrated that SnoN can also localize in the cytoplasm in some cell types (Zhang et al., 2003; Krakowski et al., 2005). For example, immunohistochemical analysis of breast carcinoma revealed the presence of cytoplasmic SnoN in certain histology types (Zhang et al., 2003). Cytoplasmic SnoN was also observed by indirect immunofluorescence in normal and non-malignant breast tissues, while the malignant tissues examined in this study showed exclusive nuclear staining of SnoN (Krakowski et al., 2005). The nuclear localization signal was mapped to lysines 30 and 31, as mutations of these lysine residues caused exclusion of SnoN from the nucleus in mouse fibroblast cells (Krakowski et al., 2005), although the mechanism of translocation were not clear from this study. The presence of cytoplasmic SnoN may offer an alternative mechanism by which SnoN affects transcription through sequestration of SnoN binding proteins (see Section 1.4.1.1).

1.4 SnoN as a transcriptional regulator

SnoN can associate with chromatin through the Smad signal transducer proteins, suggesting SnoN can act as a transcriptional regulator. In many cases, SnoN acts as a transcriptional corepressor, but may also function as a coactivator in certain cell types (Sarker et al., 2005). Particularly, SnoN has versatile functions in the TGF-β signalling pathway.

1.4.1 SnoN as a transcriptional repressor

SnoN is believed to repress transcription via various mechanisms. The N-terminal v-Ski homology domain of SnoN can bind $TAF_{II}110$, an activator of the transcription factor Sp1, suggesting that SnoN may function as a repressor through sequestration of transcriptional coactivators (Cohen et al., 1998). However, Cohen et al (1998) also found that a Gal4-SnoN fusion protein repressed transcription in a heterologous reporter assay, indicating that SnoN may also function as a general corepressor independent of the promoter context. Later on, SnoN was shown to associate with the nuclear hormone receptor corepressor (N-coR) and mSin3A, components of the histone deacetylase (HDAC) complex, indicating that SnoN may recruit the HDAC complex to specific promoters to repress transcription (Nomura et al., 1999). Accordingly, SnoN sequestration by microinjection of anti-SnoN antibody significantly impaired the repressor functions of several transcription factors that utilizes the HDAC activity for gene silencing, including Mad, thyroid hormone receptor-beta, retinoblastoma (Rb) protein and Glioblastoma-3 (Gli3) protein (Nomura et al., 1999; Tokitou et al., 1999; Dai et al., 2002). Altogether, these observations point to a role of SnoN as a transcriptional corepressor.

1.4.1.1 SnoN in TGF-β signalling pathway

The biological implication of SnoN corepressor function was realized through the ability of SnoN to antagonize the TGF-β signalling pathway (Stroschein et al., 1999). The transforming growth factor (TGF)-β family of cytokines plays pleiotropic roles in tissue development and homeostasis of metazoans by controlling a myriad of cellular processes including cell proliferation and extracellular matrix (ECM) remodeling

(Massague, 1998). The prominent function of TGF-β is its antiproliferative effect on epithelial cells, consistent with the role of TGF-β as a tumor suppressor. TGF-β signalling cascade is initiated upon binding of the TGF-β ligand to the high-affinity TGF-β type II serine/threonine kinase receptor (TβRII), which recruits and phosphorylates the type I serine/threonine kinase receptor (TβRI), forming an active heteromeric receptor signalling complex (Massague, 2000) as shown in Figure 1-3. Once activated, the type I kinase receptor binds and phosphorylates members of the Smad family of proteins at the last two serine residues in the SXS motif (Massague, 2000). The receptor-regulated Smad (R-Smad) subfamily and the common-partner Smad (Smad4) play a major role in transducing the signal from the activated TGF-β receptors at the cell surface to the nucleus (Massague, 2000). In the nucleus the ligand-phosphorylated-R-Smads (Smad2 or Smad3) in complex with Smad4 bind to Smad Binding Elements (SBE) within promoters of TGF-β-responsive genes and in collaboration with distinct transcriptional coregulators induce or block the transcription of these genes (Reguly and Wrana, 2003). For instance, the Smad complex can induce gene expression by recruiting the transcriptional coactivators p300 and CBP (Janknecht et al., 1998), or repress gene expression by recruiting the HDAC corepressor complex (Liberati et al., 2001; Kang et al., 2005).

The TGF-β-Smad signalling pathways can be negatively regulated via several different mechanisms. The dephosphorylation of R-Smads by PPM1A/PP2Cα phosphatase promotes the nuclear export of R-Smads and may present a potential mechanism for terminating TGF-β signalling (Lin et al., 2006). In addition, TGF-β can engage its own negative feedback mechanism by upregulating the transcriptional regulator SnoN (Stroschein et al., 1999). The ability of SnoN to associate with DNA

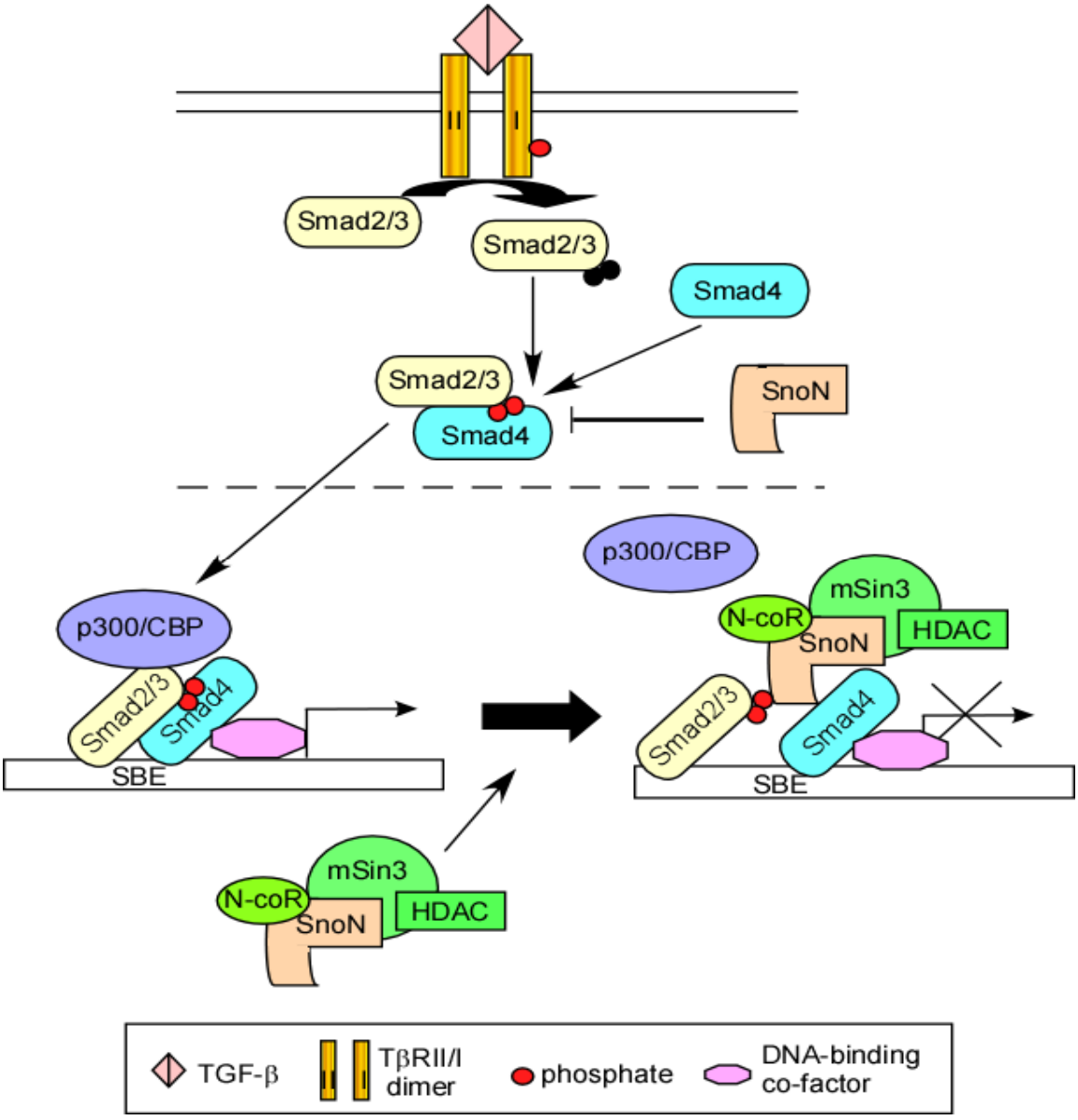
through the interaction of SnoN with Smad2/3/4 complex suggests that SnoN may act via the TGF-β-Smad signalling pathways to regulate cellular responses. When overexpressed, SnoN antagonizes the ability of TGF-β to induce transcription (Stroschein et al., 1999; Sun et al., 1999b). SnoN-inhibition of TGF-β-dependent transcription may occur via various mechanisms (Figure 1-3). SnoN can disrupt the transcriptionally active heteromeric Smad complex in the nucleus by binding to the same interaction site between Smad2/3 and Smad4 (Wu et al., 2002). SnoN can also recruit a histone deacetylase complex (HDAC) to the Smads-bound promoters of TGF-β responsive genes (Akiyoshi et al., 1999; Luo et al., 1999). Alternatively, cytoplasmic SnoN can sequester Smad proteins in the cytoplasm, thus inhibiting TGF-β signals (Krakowski et al., 2005).

1.4.2 SnoN as a transcriptional activator

Recent research from our laboratory has shown that at physiological concentrations, SnoN mediates TGF-β-induced transcription. Using the RNA interference (RNAi) method of gene-silencing, Sarker et al (2005) found that knockdown of endogenous SnoN in Mv1Lu cells, a cell type widely-used in TGF-β signalling studies, abolishes specifically the TGF-β-dependent transcriptional activation while exhibiting no effect on the TGF-β-dependent transcriptional repression. Interestingly, these effects of SnoN were found to be cell type-dependent (Sarker et al., 2005). These findings suggest that under certain circumstances, SnoN activates rather than represses TGF-β-induced transcription. The exact mechanism by which SnoN plays distinct roles in transcription remains to be elucidated.

Figure 1-3 SnoN inhibition of the TGF-β Signalling pathway

TGF-β ligand binding promotes the assembly of heteromeric ligand-receptor complex where the Type II TGF-β serine/threonine kinase receptor (TβRII) phosphorylates and activates the type I serine/threonine kinase receptor (TβRI). The activated TβRI then binds and phosphorylates the TGF-β-regulated Smad2/3 on the last two serine residues of the SSXS motif in their coserved C-terminus. The phosphorylated Smad2/3 associates with Smad4 forming a heteromeric complex that accumulates in the nucleus. The R-Smad-Smad4 complex can transactivate gene expression in the nucleus by recruiting p300/CBP coactivators. Nuclear SnoN can inhibit Smad transactivation by recruiting the HDAC complex and disrupting the Smad coactivator complex. Cytoplasmic SnoN may sequester the Smad proteins in the cytoplasm, thus preventing gene transcription. (adapted from Reguly and Wrana, 2003; Luo, 2004)


II
I
Smad2/3
Smad2/3
Smad4
Smad2/3
Smad4
SnoN
p300/CBP
Smad2/3
Smad4
SBE
p300/CBP
mSin3
N-coR
SnoN
HDAC
Smad2/3
Smad4
SBE
mSin3
N-coR
SnoN
HDAC
TGF-β
TβRII/I dimer
phosphate
DNA-binding co-factor

1.5 Biological functions of SnoN

1.5.1 Cell proliferation

Various lines of evidence indicate that SnoN can regulate cell proliferation. SnoN expression is regulated during cell cycle progression, cell cycle entry and exit (Mimura et al., 1996; Pearson-White and Crittenden, 1997; Pearson-White et al., 1995; Wan et al., 2001). The *sno* gene, located at chromosome 3q26, is frequently amplified in several types of tumors including melanomas, breast and esophageal carcinomas (Imoto et al., 2001; Zhang et al., 2003), suggesting a role for SnoN in tumorigenesis. Consistent with the idea that amplified SnoN has oncogenic effects, overexpression of SnoN in chicken embryo fibroblasts induces morphological transformation and clonal growth in soft agar assays (Boyer et al., 1993). Overexpression of SnoN in those tumors mentioned above partly explains their resistance to TGF-β-induced inhibition of cell proliferation. One possible mechanism is that SnoN may repress Smad-dependent transcription of cell cycle regulatory genes such as $p15^{INK4b}$ and $p21^{CIP1}$ that are induced by TGF-β (Liu et al., 2001). In addition, SnoN has been shown to interfere with Smad-dependent repression of c-myc, which downregulates $p21^{CIP1}$ expression (Edmiston et al., 2005). Together, these data support the idea that SnoN is a proto-oncogene.

Despite several studies on the role of overexpressed SnoN, little is known about the role of endogenous SnoN. Studies utilizing targeted disruption of the *sno* gene have revealed puzzling phenotypes. $sno^{+/-}$ heterozygote mice have higher rates of spontaneous and carcinogene-induced tumors as compared to the wild type controls (Shinagawa et al., 2000). Moreover, $sno^{+/-}$ mouse embryonic fibroblasts display increased rates of cell proliferation as compared to the wild type control (Shinagawa et al., 2000). These two

observations suggest the possibility that SnoN, at its endogenous concentrations, could act as a tumor suppressor, while overexpressed SnoN has tumor promoting functions. However, two studies using *sno* knockout mice have reported different roles of SnoN. In the study by Shinagawa et al (2000), *sno*$^{-/-}$ mice exhibited embryonic lethality due to defects in blastocyst formation, thus precluding further studies on tumorigenecity of SnoN, but pointing to a possible role in early embryo development. On the other hand, Pearson-White and McDuffie (2003) reported viable *sno*$^{-/-}$ mice with only defective T-cell activation due to increased sensitivity to TGF-β. The reason for the differences observed in the two *sno*$^{-/-}$ mice studies is unclear. Interestingly, knockdown of endogenous SnoN by RNAi significantly impaired TGF-β-induced cell cycle arrest in lung epithelial cells, suggesting that SnoN is required for the antiproliferative effect of TGF-β in certain cell types (Sarker et al., 2005). These studies indicate that endogenous SnoN has a complex role in regulating cell proliferation that is context-dependent. Thus, SnoN may have distinct tumor promoting or tumor suppressing roles depending on the level of SnoN within the cells as well as cell types.

In conclusion, SnoN appears to positively or negatively regulate cell proliferation in a manner dependent on its cellular concentration and cell types. These findings support the idea that SnoN may act as a tumor suppressor or tumor promoter. Further studies are required to better define these roles and understand the mechanism by which they occur.

1.5.2 Cell differentiation

While much of the emphasis on SnoN function has been on its role in cell proliferation and transformation, SnoN also functions in cell differentiation. In

particular, SnoN has been suggested to modulate the differentiation of skeletal muscle cells. High level of SnoN induces terminal myogenic differentiation in quail embryo cells similar to Ski (Boyer et al., 1993). Consistent with a role of SnoN in muscle differentiation, transient upregulation of SnoN mRNA is observed at the first 12 hours of myogenesis in C2C12 mouse myoblast cells (Mimura et al., 1996). Sno transcript is also found to be elevated in embryonic and neonatal skeletal muscle but decreased in adult skeletal muscle in mice (Pelzer et al., 1996; Pearson-White and Crittenden, 1997). These findings suggest that SnoN may play a role in myoblast functions during myogenesis.

In addition to a role for SnoN in muscle differentiation, SnoN has been implicated specifically in the central nervous system. SnoN has been demonstrated to promote the growth of axons in differentiated cerebellar granule neurons (Stegmuller et al., 2006). RNAi knock-down of SnoN significantly impaired granule neuron parallel fibre formation, suggesting a critical role of SnoN in the development of the mammalian brain (Stegmuller et al., 2006).

Thus, the emerging picture is that SnoN acts as a versatile modulator of diverse biological responses. As a result, there is a great interest in elucidating the mechanisms that regulate SnoN function in these responses. To date, these mechanisms remain incompletely understood.

1.6 Modulation of SnoN function

The expression of SnoN is tightly controlled by TGF-β. Transcription of SnoN is upregulated within two hours of TGF-β stimulation, possibly to participate in a negative feedback mechanism (Stroschein et al., 1999; Sun et al., 1999b). Recent studies indicate that regulation of SnoN transcription is modulated independently by Smad2 and Smad3

(Zhu et al., 2005). Examination of SnoN promoter structure reveals two TGF-β responsive elements, the Smad-binding element (SBE) and the TGF-β inhibitory element (TIE) (Zhu et al., 2005). Occupancy of the SBE by a Smad2/4 complex activates SnoN transcription, whereas occupancy of the TIE by a Smad3/4 complex represses SnoN transcription in mouse AKR-2B and rat NRK fibroblast cells (Zhu et al., 2005).

In addition to SnoN transcriptional regulation by TGF-β, SnoN is rapidly degraded via the ubiquitin-proteasome pathway upon TGF-β stimulation, and this TGF-β-induced polyubiquitination of SnoN is mediated by the Smad2/3-dependent recruitment of two distinct E3 ubiquitin ligases, Smurf2 and APC^{Cdh1} (Bonni et al., 2001; Stroschein et al., 2001; Wan et al., 2001). Polyubiquitination of SnoN via the two E3 pathways requires lysines 440, 446 and 449 (Stroschein et al., 2001). In addition, the Destruction-box (D-box) motif (RxxLxxxN, residues 164-172) in SnoN is essential for the APC^{Cdh1}-dependent degradation (Wan et al., 2001; Stroschein et al., 2001). The R-Smads, therefore, serve as crucial scaffolds for the multimeric ubiquitination complex (Bonni et al., 2001; Stroschein et al., 2001). In view of these observations, TGF-β-induced nuclear accumulation of R-Smads results in increased interactions between SnoN and the R-Smads, and consequent degradation of SnoN by ubiquitin-proteasome pathway (Bonni et al., 2001).

1.7 Sumoylation in the regulation of transcription

Ubiquitination of SnoN demonstrates an important post-translational regulatory mechanism of SnoN. Therefore, other such modifications of SnoN may present potential mechanisms to explain distinct functions of SnoN. Interestingly, a number of transcriptional modulators, including the myocyte enhancer factor-2A (MEF2A) and the

Smad interacting protein-1 (SIP1), can activate or repress transcription depending on the posttranslational modification status of the protein. Sumoylation, in particular, has emerged as a key modification in regulating transcription factors including MEF2A, SIP1 as well as the GC-box-binding Specificity protein-3 (Sp3), that have both transcriptional activator and repressive functions (Ross et al., 2002; Sapetschnig et al., 2002; Long et al., 2005; Riquelme et al., 2006; Shalizi et al., 2006).

1.7.1 The sumoylation machinery

The small ubiquitin-related modifier (SUMO) shares only 18% sequence identity to ubiquitin, but its N-terminal structure is virtually superimposable to ubiquitin (Johnson, 2004). Unlike ubiquitination, however, sumoylated proteins are not targeted for degradation. Instead, sumoylation appears to modulate protein properties including subcellular localization, protein-protein interactions, protein stability, and transcriptional activities (Johnson, 2004).

SUMO is synthesized as a precursor protein that requires the C-terminal hydrolase activity of a SUMO protease (SENP) to be processed into the mature SUMO revealing the last glycine residue at the C-terminus(Gill, 2004). SUMO, in most cases, is attached to the substrate on the lysine residue that is contained in the short consensus sequence ΨKXE/D, where Ψ is a large hydrophobic residue; K is the SUMO-acceptor lysine; X is any amino acid; and E/D is glutamate or aspartate. The conjugation of SUMO onto the substrate occurs via the formation of an isopeptide bond between the carboxyl group of the glycine in SUMO and the ε-amine of the lysine in the SUMO consensus motif of the substrate.

The process of sumoylation is analogous to ubiquitination, and occurs via an ATP-dependent enzyme cascade involving the heterodimeric SUMO activating enzyme (E1), Aos1/Uba2; the SUMO conjugating enzyme (E2), Ubc9; and one of a number of SUMO ligases (E3) which facilitate the transfer of the SUMO moiety from Ubc9 to its substrates (Figure 1-4). The E3 SUMO ligases discovered to date belong to one of three classes of proteins: RanBP2 (Ran-binding protein 2) nucleoporin, PIAS (protein inhibitor of activated STAT) family proteins, and Pc2 (polycomb group protein 2) (Johnson, 2004). Sumoylation is a reversible process. Desumoylation is achieved by a class of SUMO-specific proteases (SENPs) which hydrolyze the isopeptide bond between SUMO and the substrate, adding to the dynamic nature of this modification.

1.7.2 Sumoylation as a modulator of transcription factors

Sumoylation may regulate signalling pathways at various levels, ultimately leading to changes in gene expression. The PIAS family proteins, whose members include PIAS1, PIAS3, PIASxα, PIASxβ and PIASy, are transcription factors as well as SUMO E3 ligases, linking sumoylation directly to transcriptional regulation. The molecular mechanism by which sumoylation regulates transcription is presently unclear but appears to involve a combination of events including subcellular targeting, protein-protein interaction and protein stability. In many cases, sumoylation has been associated with transcriptional repression (Muller et al., 2004). One possible mechanism of SUMO-dependent repression is that sumoylated transcription regulators recruit the HDAC corepressor complex to the target promoters resulting in deacetylation of histones and transcription repression. For example, p300, which in most cases acts as transcription coactivator, can repress transcription when sumoylated on its Cell Cycle Regulatory

domain 1 (CRD1), by recruiting HDAC6 (Girdwood et al., 2003). Targeting of transcription factors to nuclear bodies provides another interesting mechanistic model in which chromatin regulatory complexes may be assembled in a SUMO-dependent manner. The PIAS proteins contain the SAP (SAF-A, Acinus, PIAS) domain that associates with the nuclear matrix, part of which overlaps with the promyelocytic leukaemia nuclear bodies (PML NBs). Re-distribution of PIAS substrates such as LEF1 to nuclear bodies has been associated with transcriptional modulation (Sachdev et al., 2001).

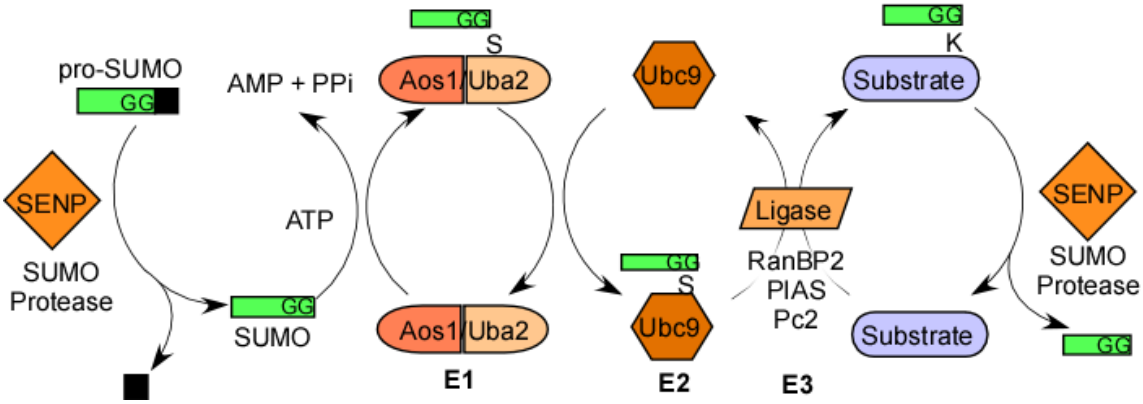


Figure 1-4 The sumoylation pathway

SUMO is synthesized as a precursor protein that is C-terminally cleaved (black rectangle represents the cleaved propetide sequence HSTVN) by a SUMO protease (SENP) to reveal the Gly-Gly motif for conjugation. Mature SUMO protein is conjugated in an ATP-dependent reaction via a thioester (S) bond to the heterodimeric E1 activating enzyme, Aos1/Uba2. The SUMO moiety is then transferred to the E2 conjugation enzyme, Ubc9 via a thioester (S) bond. A SUMO E3 ligase facilitates the transfer of SUMO onto its substrate via an isopeptide bond on the lysine (K) of the substrate. Desumoylation occurs through an isopeptide cleavage by a SUMO protease (SENP). (adapted from Gill, 2004)

1.8 Thesis hypothesis and aims

The models of SUMO-dependent transcription modulation described in Section 1.7.2 may provide some explanation for the sometimes paradoxical roles of SnoN. Interestingly, by sequence analysis and alignment I found two putative sumoylation consensus sequences which are evolutionarily conserved in human, mouse and chicken SnoN proteins (Figure 1-5). It is reasonable to consider that SnoN may be a potential target of sumoylation.

Therefore, I proposed the following hypothesis and specific aims for this thesis project:

Hypothesis:

SnoN is a target of sumoylation and SUMO modification of SnoN alters SnoN functions

Specific aims:

1) **Identify and characterize SnoN post-translational modification by SUMO**
2) **Determine the role of SnoN sumoylation on SnoN functions**

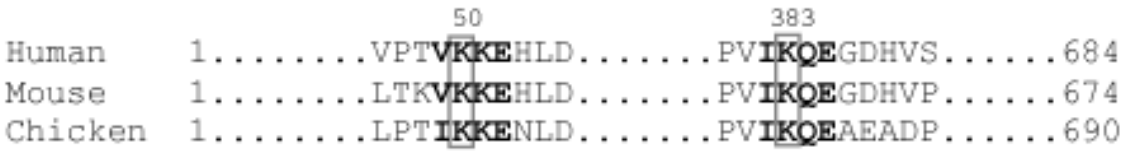


Figure 1-5 Partial sequence alignment of the human, mouse and chicken SnoN proteins, showing potential sumoylation consensus sites

The regions containing the two SUMO-consensus motifs are shown in bold letters with the putative lysine acceptor sites in rectangular boxes. The amino acid positions of these lysine residues in the human SnoN sequence are indicated.

Chapter Two: Materials and Methods

2.1 Cell lines, culturing media and conditions

All cell lines used in this thesis project are generous gifts from Dr. J. Wrana Lab (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON). Human kidney epithelial 293T cells were cultured in Dulbecco's modified essential medium (DMEM) with high glucose and L-glutamine (Gibco) containing 10% fetal bovine serum (FBS; Gibco). To subculture, 293T cells at 80-85% confluency were rinsed once with 1x PBS, detached by incubating with 0.05% trypsin-EDTA (Gibco) for 30 sec and passaged at 1:6 to 1:8 dilutions. Mouse myoblast C2C12 cells were maintained in DMEM containing high glucose, L-glutamine and sodium pyruvate (Gibco) supplemented with 10% FBS. C2C12 cells reaching 70-80% confluency were subcultured. The cells were washed once with PBS, trypsinized at 37ºC for up to 45 sec and passaged at 1:6 to 1:8 dilutions. Human hepatoma HepG2 and mink lung epithelial Mv1Lu cells were grown in minimum essential medium (MEM; Gibco) containing 1% nonessential amino acids (NEAA; Gibco) and 10% FBS. HepG2 cells were subcultured at 80-85% confluency. Following two washes with PBS, HepG2 cells were trypsinized at 37ºC for 3 to 4 min, collected into a 50-ml conical tube containing the culturing medium and centrifuged in a Sigma 4-15 table top centrifuge (Sigma) at 1000 rpm for 5 min. The cell pellet was resuspended in fresh medium by passing through an 18"-gauge syringe needle for 5 to 6 times, and passaged at 1:3 dilutions. To subculture Mv1Lu cells, 80-85% confluent cells were washed twice with PBS, trypsinized at 37ºC for 2 to 3 min and passaged at 1:7 dilutions.

All cell lines were incubated at 37ºC under a 5% carbon dioxide atmosphere, and confirmed to be free of mycoplasma contamination. The characteristics and experimental applications of each cell type in this study are summarized in Table 2-1.

Table 2-1 Cell lines and descriptions

Cell lines	Species	Tissue	Properties & applications for this study
293T	human	kidney, embryonic, epithelial	▪ High transfection efficiency ▪ Widely used for overexpression and immunoprecipitation experiments
C2C12	mouse	muscle, adult, myosatellite	▪ Skeletal muscle differentiation model ▪ Express muscle-specific proteins ▪ Myogenesis-induction signalling reporter assays
HepG2	human	liver, adolescent, hepatoma	▪ Good transfection efficiency ▪ Has a robust TGF-β signalling pathway ▪ Widely-used for TGF-β signalling reporter assays
Mv1Lu	mink	lung, near-term fetus, epithelial	▪ Has a well-characterized functional TGF-β signalling pathway ▪ Widely-used for TGF-β signalling reporter assays

2.1.1 Cell transfection

2.1.1.1 Calcium phosphate precipitation method

293T and HepG2 cells were transfected using the widely-used calcium phosphate method (Graham and Van der Eb, 1973). One day after seeding, the cells were fed fresh media such that it made up 90% (v/v) of the total transfection solution 3 to 4 hours before transfection. The expression plasmids DNA were mixed in a solution containing a final concentration of 125 mM calcium chloride at room temperature. An equal volume of room-temperature 2x HEPES-buffered saline (HeBS) was added drop-wise to the DNA

contents for each transfection. The resulting transfection solution was agitated by bubbling and vortexing for 10 sec, and incubated at room temperature for 20 min before applying onto cells in a biosafety cabinet (ll). After 16 h of incubation, cells were washed once (for 293T cells) or twice (for HepG2 cells) with fresh regular growth media.

2.1.1.2 Fugene 6™ Transfection Kit (Roche Applied Biosciences)

C2C12 and Mv1Lu cells were transfected using a liposome-based FuGene® 6 Transfection Reagent according to the manufacturer's instructions (Roche Applied Biosciences), with the following specifications: cells were transfected using 1:3 ratio of DNA (μg):FuGene®(μl) mixture and incubated in the transfection solution for 20 to 22 h.

2.2 Bacteria strains and culturing media

Escherichia coli DH5α™ (Invitrogen; F^- ϕ80*lac*ZΔM15 Δ[*lac*ZYA-*arg*F]U169 *deo*R *rec*A1 *end*A1 *hsd*R17[r_k^-, m_k^+] *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) bacteria were used for cultivating and maintaining the plasmids of interest. LB broth [20% Peptone (EM Sciences), 10% yeast extract (EM Sciencs), 10% NaCl] containing 100 μg/ml ampicillin (EM Sciences) was used as the liquid culturing medium. LB-ampicillin-agar [LB broth, 100 μg/ml ampicillin, 30% agar (Invitrogen)] plates were used as the solid culturing medium.

2.3 Common Buffers and Reagents

2x HeBS: 50mM HEPES [*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*; Invitrogen], 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose (Calbiochem), adjusted to pH to 7.05 with NaOH

10x PBS: 137 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4

10x SDS-PAGE Running Buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS

50x TAE: 2 M Tris, 5.71% glacial acetic acid, 50 mM EDTA, pH 8

10x TBS: 200 mM Tris, 1.37 M NaCl, adjusted to pH 7.6 with HCl

1x TBS-T: 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween-20 (Sigma)

1x Transfer Buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol

8x SDS sample buffer: 8% SDS, 400 mM Tris, pH 6.8, 40% glycerol, 400 mM DTT, 0.1% Bromophenol Blue.

4x Separating Buffer: 1.5 M Tris, adjusted to pH 8.8 with HCl

4x Stacking Buffer: 0.5 M Tris, adjusted to pH 6.8 with HCl

100 mM Sodium phosphate buffer: 82 mM Na_2HPO_4, 18 mM NaH_2PO_4, pH 7.5

TGF-β1 (10 μM, reconstituted): 10 μg TGF-β1 (R&D Systems) in 40μl solution containing 30% acetonitrile (Sigma) and 0.1% trifluoroacetic acid (Fluka).

2.4 Plasmids

2.4.1 Generation of Sumoylation-deficient Mutants of SnoN by site-directed mutagenesis

The CMV-promoter-based pCMV5B vector plasmids containing cDNA encoding human SnoN sumoylation-deficient mutant proteins – SnoN (K50R), SnoN (K383R) and SnoN (K50/383R, hereafter designated KdR) were constructed by site-directed mutagenesis using the method of splicing by overlap extension (SOEing) PCR, as illustrated in Figure 2-1, with the primers and templates listed in Table 2-2. In each of the replicate PCR reactions, 50 ng of template plasmid DNA was mixed with 500 nM of each primer, 200 μM dNTPs (Invitrogen), and 1 U *Pwo* DNA polymerase (Roche) in 1x reaction buffer to a final volume of 50 μl. Two separate reactions, one with no template

Figure 2-1 Schematic of site-directed mutagenesis by the PCR method

Primers (black horizontal arrows, see Table 2-2 for sequences), shown relative to the indicated lysine sites, were used to generate PCR fragments. For K50R and KdR mutations, a second PCR step (SOEing PCR) was required to produce the final PCR fragments. Vertical lines between PCR fragments indicate overlapping complementary sequences. The black diamond represents the resulting lysine to arginine mutation(s). The PCR inserts containing *Sal*I and *BstE*II sites were subcloned into the *Sal*I/*BstE*II-digested pCMV5B/FLAG/SnoN(wt) vector backbone (black rectangles represents the relative positions of the two lysine sites).

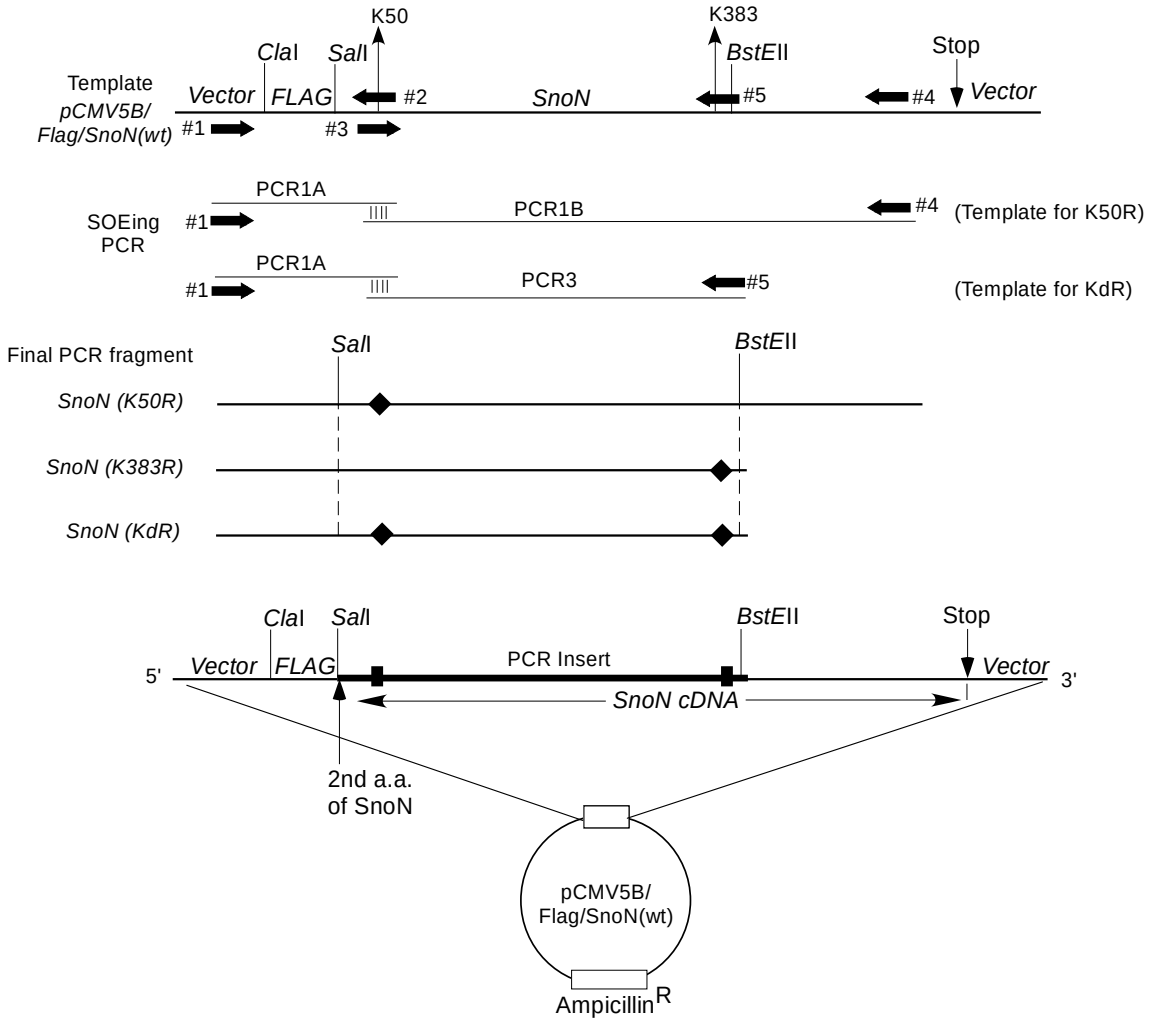

K50
K383
ClaI
SalI
BstEII
Stop
Template
pCMV5B/
Flag/SnoN(wt)
Vector
FLAG
#2
SnoN
#5
#4
Vector
#1
#3
PCR1A
PCR1B
#4
SOEing
PCR
#1
(Template for K50R)
PCR1A
PCR3
#5
#1
(Template for KdR)
Final PCR fragment
SalI
BstEII
SnoN (K50R)
SnoN (K383R)
SnoN (KdR)
ClaI
SalI
BstEII
Stop
5'
Vector
FLAG
PCR Insert
Vector
3'
SnoN cDNA
2nd a.a.
of SnoN
pCMV5B/
Flag/SnoN(wt)
AmpicillinR

Table 2-2 Templates and primers used for site-directed mutagenesis

Desired mutation(s)	Templates	SOEing PCR Primers
SnoN (K50R)	pCMV5B/FLAG/SnoN(wt)	PCR1A pCMV5,5'A-5' CTCGTTTAGTGAACCGTCAG 3' (#1) hSonN,302-5' CAAGTGTTCCTTCCTAACTGTTGGCAC 3' (#2)
	pCMV5B/FLAG/SnoN(wt)	PCR1B hSnoN,402-5' GTGCCAACAGTTAGGAAGGAACACTTG 3' (#3) mSnoN,103-5' GCTTTTTCTCTAAGTCTCTCTGTTC 3' (#4)
	SOEing PCR1A and PCR1B	pCMV5,5'A-5' CTCGTTTAGTGAACCGTCAG 3' (#1) mSnoN,103-5' GCTTTTTCTCTAAGTCTCTCTGTTC 3' (#4)
SnoN (K383R)	pCMV5B/FLAG/SnoN(wt)	pCMV5,5'A-5' CTCGTTTAGTGAACCGTCAG 3' (#1) hSnoN,303-5' CATGGTCACCTTCCTGCCTTATAACAGGATAC 3' (#5)
SnoN (KdR)	pCMV5B/FLAG/SnoN(wt)	PCR3 hSnoN,402-5' GTGCCAACAGTTAGGAAGGAACACTTG 3' (#3) hSnoN,303-5' CATGGTCACCTTCCTGCCTTATAACAGGATAC 3' (#5)
	SOEing PCR3 and PCR1A	pCMV5,5'A-5' CTCGTTTAGTGAACCGTCAG 3' (#1) hSonN,303-5' CATGGTCACCTTCCTGCCTTATAACAGGATAC 3' (#5)

and the other with no primers, were used as negative controls. The reaction solution was subjected to temperature cycles consisting of one cycle of 5 min at 93°C (melting); 30 cycles of 30 sec at 93°C (melting), 30 sec at 55°C (annealing) and 2 min at 72°C (extension); and one cycle of 10 min at 72°C (extension) in a PTC-200 Thermal Cycler (MJ Research; Dr. S. Robbins, University of Calgary, AB). 5 µl of the reaction products was separated on 1.2% agarose gels containing 1 µg/ml ethidium bromide (Invitrogen) at 90V in 1x TAE buffer using a Mini-Sub® Cell GT electrophoresis apparatus (Bio-Rad Laboratories). The gels were imaged using a VersaDoc 5000 Imager (Bio-Rad Laboratories) to check for amplification products (Figure 2-2A).

2.4.1.1 SnoN (K383R) mutagenesis

The PCR product resulted from the K383R mutagenesis reaction were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) by vigorous shaking for 15 sec followed by centrifugation at 14,000 rpm for 3 min. The aqueous (upper) layer was carefully transferred to a fresh microfuge tube, precipitated in one-tenth volume of 3 M potassium acetate (pH 5.2) and 3 volumes of 95% ethanol overnight at -20°C. The precipitated DNA was pelleted by centrifuging at 14,000 rpm for 12 min at 4°C. The pellet was washed twice with 1 ml of 70% ethanol, air-dried, and re-dissolved in 40 µl sterile double distilled water. Subsequent steps were carried out as detailed in Section 2.4.1.3.

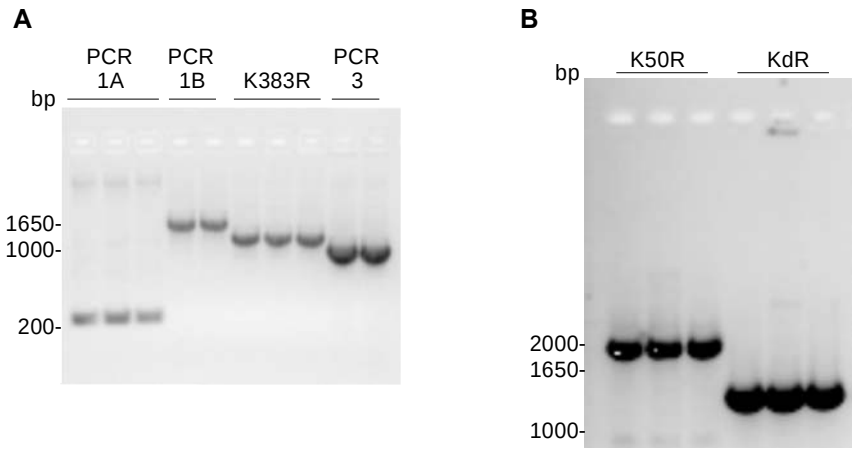


Figure 2-2 PCR amplification of site-directed mutagenesis products

A. The template, pCMV5B/FLAG/SnoN(wt), was used to generate the indicated PCR fragments. **B**. Amplification by SOEing PCR. K50R and KdR fragments were amplified using the overlapping PCR templates, PCR1A/PCR1B and PCR1A/PCR3, respectively. 10% of each PCR reaction replicate was resolved on a 1.2% agarose gel. All PCR reactions amplified products of the expected sizes.

2.4.1.2 SnoN (K50R) and SnoN (KdR) mutagenesis

For K50R and KdR mutagenesis, the desired products generated from PCR1A, PCR1B and PCR3 were gel-extracted using the GENECLEAN® kit according to the manufacturer's instruction (Qbiogene) as follows. The desired band was excised and dissolved in 3 volumes of NaI solution at 55°C. The resulting clear solution was incubated on a rocker for 15 min with 10 µl GlassMilk® suspension to bind DNA. The mixture was centrifuged at 14,000 rpm for 5 sec at room temperature. The pellet was resuspended in the washing solution and the mixture was re-centrifuged. This step was repeated two more times. Following the final wash and removal of supernatant, the pellet was dried at 55°C for 2 min. The GlassMilk® pellet was resuspended in 20 µl sterile double distilled water to elute DNA and then centrifuged at 14,000 rpm for 30 sec. The supernatant containing the eluted DNA was collected into a fresh tube.

A second PCR step was performed using the gel-purified products isolated in the previous step as templates and the appropriate primers in the same PCR conditions as described in Section 2.4.1. The expected amplification products were detected (Figure 2-2B). The final PCR fragments were purified by the phenol:chloroform extraction method as described in Section 2.4.1.1.

2.4.1.3 DNA ligation and transformation into DH5α™

The final PCR DNA products and the pCMV5B/FLAG/SnoN(wt) plasmid were digested first with *BstE*II restriction enzyme and subsequently with *Sal*I for 2 h each according to the manufacturer's specifications (New England Biolab). The digested vector was treated with a calf intestinal alkaline phosphatase (Roche Applied Biosciences) to minimize self-ligation. Both the digested PCR inserts and the vector

were gel-extracted using the GENECLEAN® kit. Equimolar concentrations of the vector and each insert were ligated a T4 DNA ligase (New England Biolab) at 16°C in a Hakke G circulating water bath (Hakke; Dr. C. Brown, University of Calgary, AB) for 16-20 h.

The ligation products were transformed into *E. coli* DH5α™ cells. The resulting colonies were inoculated in 5 ml LB-ampicillin broth at 37°C for 16 h in a shaking incubator (Forma Scientific; Dr. T. Beattie, University of Calgary, AB). The plasmids of interest were isolated using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions (Qiagen) as follow. Bacterial culture was collected in a 15-ml conical tube (Falcon) and centrifuged at 3,500 rpm for 15 min at 4ºC in a JS3.0 rotor in a Beckman J-6M induction drive centrifuge (Beckman; Southern Alberta Cancer Research Institute, AB). The pellet was resuspended in 250 ul Buffer P1 containing RNase A and transferred to a microcentrifuge tube. Then 250 ul Buffer P2 was added to the suspension to lyse the bacteria. The lysate was mixed with 350 ul neutralizing Buffer P3 and centrifuged at 13,200 rpm for 10 min at room temperature to separate the plasmid DNA from genomic DNA and proteins. 750 ul of the supernatant was passed through a QIAprep column using a vacuum manifold (Qiagen). The column was washed once with 750 ul Buffer PB, and then centrifuged at 13,200 rpm for 1 min to remove residual buffer. The bound plasmid DNA on the membrane of the column was eluted by adding 40 ul warm (50ºC) double distilled water and centrifuging at 13,200 rpm for 1 min. The elution step was repeated once to maximize DNA recovery. Once the plasmid of interest was isolated, the mutation(s) in each construct was verified by nucleic acid sequencing (University of Calgary DNA Core Sequencing Facility, AB).

2.4.2 Generation of SUMO1-SnoN fusion protein expression construct

Generation of a pCMV5B plasmid encoding SUMO1-SnoN fusion protein and subsequent studies using this expression construct were carried out in collaboration with Dr. Isabelle Pot. The insert containing the SUMO1 cDNA with flanking 5' *Cla*I and 3' *Sal*I sites was derived from PCR using the primers and template listed in Table 2-3. The resulting fragment was cut with *Cla*I and *Sal*I, gel-extracted, and subsequently ligated into the *Cla*I/*Sal*I-digested and gel-purified pCMV5B/FLAG/SnoN(wt) vector to replace the FLAG tag with the SUMO1 sequence (Figure 2-3).

Table 2-3 Template and primers used for constructing the *Cla*I-SUMO1-*Sal*I insert

Template	pCMV5/HA/SUMO1
Primers	hSUMO1, 201-5' CCATCGATGGATCCATGTCTGACCAGG 3' hSUMO1, 101-5' CGACGTCGACCCGTTTGTTCCTGATAAACTTC 3'

2.5 Protein Analysis

2.5.1 Cell Extract Preparation

Two days post-transfection, cells were rinsed once with 1x PBS, and lysed in TNTE lysis buffer [50mM Tris, 150mM NaCl, 1mM EDTA, 0.5% (v/v) Triton-X-100] containing freshly added protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 10 µg/ml pepstatin A (Sigma), 100 µg/ml trypsin inhibitor (Roche), 100 µg/ml benzamidine hydrochloride (Calbiochem), 10 µg/ml antipain (Roche) and 50 µg/ml aprotinin (Roche)] for 15 min at 4ºC on a VWR S-500 orbital shaker (VWR). Cell lysates were centrifuged at 14,000 rpm for 10 min at 4ºC in a Sigma 1-15 microcentrifuge (Sigma). A small fraction of the supernatant was saved for Bradford-based protein assays

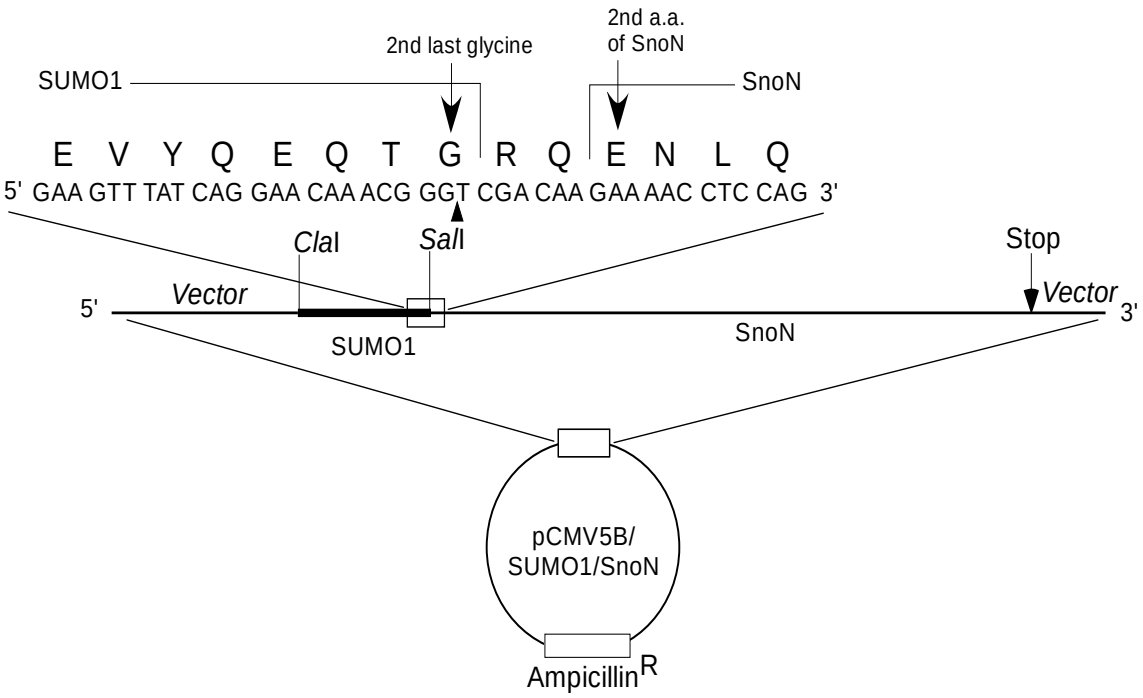


Figure 2-3 Schematic of SUMO1-SnoN expression construct

PCR-derived SUMO1 fragment (see Table 2-3 for template and primers) was digested with *Cla*I and *Sal*I restriction enzymes and subcloned into the *Cla*I/*Sal*I-digested pCMV5B/FLAG/SnoN(WT) vector. The nucleotide and amino acid sequences between the SUMO1 and SnoN junction are shown. The SUMO1 insert lacks the propeptide sequence and contains only the 2nd last glyine of the diglycine (GG) motif at the C-terminus

in 96-well plates (Costar), using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories) to determine the protein concentration by measuring the absorbance at 595 nm in a microplate reader (Bio-Rad; Dr. D. Rancourt, University of Calgary, AB). The protein concentration in each sample was based on a protein standard curve that was generated by known concentrations of bovine serum albumin (BSA). The remainder of the supernatant was boiled in 1x SDS-sample buffer for 2 min.

2.5.2 Immunoprecipitation

Two days post-transfection, cells were lysed in TNTE lysis buffer containing protease inhibitors (see 2.5.1) and phosphatase inhibitors [10 mM sodium pyrophosphate (EM Sciences), 1 mM sodium orthovanadate (Alfa Aesar) and 25 mM sodium fluoride (EM Sciences)]. In addition, where appropriate, 20 mM N-ethylmaleimide (NEM; Sigma) isopeptidase inhibitor or equivalent volume of the vehicle was included in the lysis buffer. Cell lysates were centrifuged at 14,000 rpm for 10 min at 4ºC, and 10% of the supernatant volume was removed for protein concentration determination and protein expression analyses. The remainder of the supernatant was incubated at 4ºC first with the specified antibodies of interest alone (see Table 2-4 for dilutions) for 1-2 h, or overnight in the case of endogenous protein immunoprecipitation, and then together with 70 µl protein G-sepharose beads (diluted 1:5 in TNTE wash buffer; Amersham Biosciences) for 1 h. The beads were centrifuged at 3,000X g for 1 min at room temperature. Following the removal of supernatant, the beads were resuspensed in 1 ml ice-cold TNTE wash buffer containing 0.1% Triton X-100 and centrifuged again as before. This washing step was repeated 5 more times before boiling the beads in 20 to 30 µl 2x SDS sample buffer for 5 min.

In the case of double anti-FLAG immunoprecipitation, the immunocomplexes from the first precipitation were eluted in 1% SDS by boiling for 5 min to denature the protein complexes. 90% of eluate was diluted in TNTE wash buffer containing 0.1% Triton X-100 to reduce the SDS concentration to 0.1%, and subjected to a second anti-FLAG immunoprecipitation as described above, with the exception of an overnight incubation with double the amount of anti-FLAG antibody. Protein content from the remaining 10% eluate was mixed with an equal volume of 2x SDS sample buffer and used to analyze the single immunoprecipitation for comparison purposes.

2.5.3 Western Blotting

SDS-PAGE gels were cast using a Hoefer™ SE 200 series multigel caster (Amersham Biosciences). A 7.5% separating gel solution consists of 0.1% SDS, 7.5% acrylamide (containing 0.2% n'n'-bis methylene acrylamide; Bio-Rad Laboratories), 0.07% ammonium persulfate (APS) and 0.05% n,n,n’n’-tetramethylethylenediamine (TEMED; Bio-Rad Laboratories) in 1x Separating Buffer. The separating gel solution was poured into the gel caster, overlaid with 1-butanol:H_2O (1:1 v/v) and allowed to polymerize for 30 min. Once polymerized, the 1-butanol:H_2O layer was rinsed off with distilled water. The stacking gel solution, consisting of 0.1% SDS, 3.3% acrylamide (containing 0.088% n'n'-bis methylene acrylamide), 0.09% APS and 0.05% TEMED in 1x Stacking Buffer, was poured on top of the separating gel, and 10- or 15-well combs were inserted. The stacking gel was allowed to polymerize for 1 h.

The protein content of each cell lysate or immunoprecipitation sample was resolved on 7.5% or 10% gels using a Hoefer™ SE 260 electrophoresis apparatus (Amersham Biosciences) in 1x SDS-PAGE running buffer at 120V through the stacking

gel and at 180V through the separating gel until the dye front has just run past the bottom of the gels. Then, the protein content of the gels was transferred onto nitrocellulose membrane (Bio-Rad Laboratories) in pre-chilled 1x Transfer buffer using a Hoefer™ TE 22 transfer unit (Amersham Biosciences) at 100V for 90 min at 4ºC. The blots were blocked with 5% skim milk in 1x TBS-T for 1-2 h at room temperature. Following two rinses in TBS-T for 10 min each, the blots were incubated overnight in the appropriate primary antibody in TBS-T containing 1% skim milk at the dilutions listed in Table 2-4, at 4ºC. The blots were then washed four times in TBS-T for 10 min each. This was followed by incubation for 1 h in horse radish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody (Amersham Biosciences) diluted at 1:10,000 in TBS-T containing 2.5% skim milk at room temperature. After five 10-min washes in TBS-T, the blots were rinsed for 1 min in ECL™ Western Blotting Detection Reagents (Amersham Biosciences) followed by signal detection using a VersaDoc 5000 Imager (Bio-Rad Laboratories). Densitometry was performed using Quantity One® software (Bio-Rad Laboratories, version 4.5.2).

Table 2-4 Primary antibody specificity and working dilutions

Antibody*	Host	Antigen(s) recognized†	Stock Conc. (mg/ml)	WB	IF	IP‡
Actin[a]	rabbit	All actins	0.4-0.8	1:3000	n	n
FLAG® M2[a]	mouse	FLAG[1]	4.3	1:3000	1:250	1µg
HA.11 (16B12)[b]	mouse	Hemagglutinin[2]	5-7	1:1000	1:250	n
9E10[b]	mouse	myc[3]	3-5	1:1000	1:250	n
Smad2/3[c]	mouse	Smad2/3	0.25	1:2000	n	n
Phospho-Smad2[d]	rabbit	Smad2 ($pSer^{465/467}$)	unknown	1:1000	n	n
SnoN (H-317)[e]	rabbit	SnoN	0.2	1:3000	1:250	1µg

*Antibodies obtained from: [a]Sigma; [b]Covance; [c]BD Transduction Laboratories; [d]Calbiochem; and [e]Santa Cruz Biotechnologies. †Epitope-tag sequences recognized: [1]DYKDDDDK; [2]YPYDVPDYA; [3]EQKLISEEDL. ‡Amount of antibody per milligram of protein. n = not used.

2.6 Protein Half-life Determination

2.6.1 Cycloheximide Blocking

$1x10^5$ 293T cells were seeded in 12-well plates. On the following day, the cells were transfected with a pCMV5B expression plasmid encoding wild-type (WT), K50R, K383R or KdR version of SnoN or an empty control vector. 40 h post-transfection, the cells were treated with 10 μg/ml cycloheximide (Calbiochem) for the indicated time periods before being processed for cell extract and western blotting as described in 2.5.1 and 2.5.3, respectively. The quantified protein density at each time point was expressed as percent of protein level at time 0 and plotted versus time of treatment with cycloheximide to determine the protein half-life.

2.6.2 ^{35}S Pulse-chase

$1x10^6$ 293T cells were seeded in 100 mm dish. The cells were transfected the next day. 20 h after transfection, the cells were detached, collected, centrifuged, and resuspended in 11 ml and divided into 2 ml/well in five 6-well plates coated with 400 μl of 0.1 mg/ml poly-L-lysine (Sigma). 40-42 h after transfection, the cells were washed twice with and incubated in cysteine- and methionine-free DMEM (Gibco) for 15 min, followed by labelling with 50 μCi [^{35}S]-methionine (Amersham Biosciences) in cysteine- and methionine-free DMEM (Gibco) for 10 min (*pulse*). The cells were washed twice in regular DMEM and then incubated in DMEM containing unlabelled methionine for the indicated time period (*chase*). Cells were lysed and subjected to immunoprecipitation as described in 2.5.2 with the exception that the beads were washed twice with RIPA buffer [50mM Tris, 150mM NaCl, 1mM EDTA, 0.5% (v/v) Triton-X-100, 1% (w/v) deoxycholate] containing 0.1% SDS and then twice with RIPA buffer without 0.1% SDS.

The protein content of the IP samples was resolved by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R-250 (40% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue R-250) for 30 min, followed by an overnight de-stain in 5% (v/v) ethanol and 5% (v/v) acetic acid. The gels were rinsed in distilled water for 10 min and placed on a blotting paper (VWR Grade 703) prior to drying in a gel dryer (Bio-Rad Laboratories) for 80 min. The dried gels were exposed on a storage phosphor screen (Amersham Biosciences) for 2 days. The signal was detected by autoradiography using a STORM™ PhosphoImager™ (Amersham Biosciences; Dr. J. MacDonald, University of Calgary, AB) and quantified using Quantity One® software (Bio-Rad Laboratories, version 4.5.2). The half-life of protein was determined by plotting the intensity of labeled SnoN at each time point as a percentage of the intensity at time 0 versus the chase time.

2.7 Indirect Immunofluorescence

4×10^4 cells were seeded in 4-chamber culture glass slides (Falcon). Cells were washed once with 1x PBS and fixed in 4% formaldehyde in PBS for 10 min. Cells were permeabilized on ice with -20ºC chilled 100% methanol for 2 min, blocked with 5% BSA for 1 h at room temperature, followed by incubation with primary antibodies diluted in 0.1% BSA for 1 h at 37ºC (see Table 2-4 for dilutions). After three 2-min washes with PBS, cells were incubated with the appropriate Cy™2 (excitation 492 nm, emission 510 nm)- and/or Cy™3 (excitation 550 nm, emission 570 nm)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) diluted 1:500 in 0.1% BSA for 30 min at room temperature. Cells were rinsed four times in PBS for 3 min each and then stained with 1 µg/ml 4'.6-diamidino-2-phenylindole (DAPI; Sigma) in PBS for 2 min.

Following two 3-min rinses in PBS, the slides were mounted in GelTol™ mounting medium (Thermo) with micro cover glass (VWR), and sealed with nail polish. The immunofluorescence slide images were captured with a Zeiss Axiovert 200M fluorescent microscope equipped with the AxioCamMRc camera (Carl Zeiss; Dr. P. Forsyth, University of Calgary, AB), and visualized with AxioVision software (Carl Zeiss, Release 4.5).

2.8 Luciferase Reporter Assay

2.8.1 TGF-β signalling reporter assay

Cells were seeded in 24-well plates at a density of $2x10^4$ cells per well and one day later were transfected with the pGL3-based luciferase reporter construct 3TP-lux, SBE4-lux, or p21-p-lux (0.1µg/well; see 3.9 for description), CMV-β-galactosidase vector (0.05ug/well) and an pCMV5B expression plasmid encoding SnoN (WT), SnoN (KdR) or SUMO-SnoN or an empty control vector. One day post-transfection, cells were washed in regular medium and 3 h later, were incubated in 0.2% FBS-containing medium in the presence or absence of 100 pM TGF-β (R&D Systems) for 16-18 h at 37ºC. Then, cells were washed once in 1x PBS and lysed in 90 µl 1x Reporter Lysis Buffer (Promega). Cells were subjected to one freeze-thaw cycle by an overnight -20ºC freezing and a 30-min room temperature thawing on a rocker. The luciferase activity, measured as relative light unit (RLU, an arbitrary unit), was assayed by mixing 10 µl of cell lysates with 25 µl of Luciferase Assay Substrate (Promega) and detected in 20 s using an Orion II microplate luminometer (Berthold Detection Systems). Another 10 µl of cell lysates were subjected to the β-galactosidase assay (measured as the absorbance at 420nm, A_{420}) by mixing with 90 µl of the substrate [1 mg/ml ONPG (*o-Nitrophenyl-β-D-*

galactopyranoside; Sigma), 1 mM $MgCl_2$, 50 mM β-mercaptoethanol in 0.1 M sodium phosphate buffer, pH 7.5] at 37ºC until A_{420} reaches between 0.1 and 0.8 above the blank control. Luciferase activity (in RLU) was normalized to β-galactosidase activity to control for variation in transfection efficiency. Each experimental condition was carried out in triplicates and the data are presented as mean (+ SEM, standard error of the mean). Experiments were repeated independently at least three times.

2.8.2 Myogenesis induction reporter assay

C2C12 cells were seeded in 24-well plates at a density of $2x10^4$ cells per well and one day later were transfected with the pGL3-based luciferase reporter construct myogenin-p-luc (0.1μg/well; see 3.10 for description), CMV-β-galactosidase vector (0.05ug/well) and an empty expression vector or one encoding SnoN (WT), SnoN (KdR) or SUMO-SnoN. C2C12 cells were maintained in growth medium (see Section 2.1) until 40 h post-transfection, at which time myogenesis was initiated by incubating 90-100% confluent cells in differentiation medium [DMEM containing 2% horse serum (Gibco)] for 2 to 4 days before lysis. The cell lysates were assayed for luciferase activity and β-galactosidase activity as described in 2.8.1. Each experimental condition was carried out in triplicates and the data are presented as mean (+ SEM). Experiments were repeated independently at least three times.

Chapter Three: Results

3.1 SnoN is modified by SUMO1

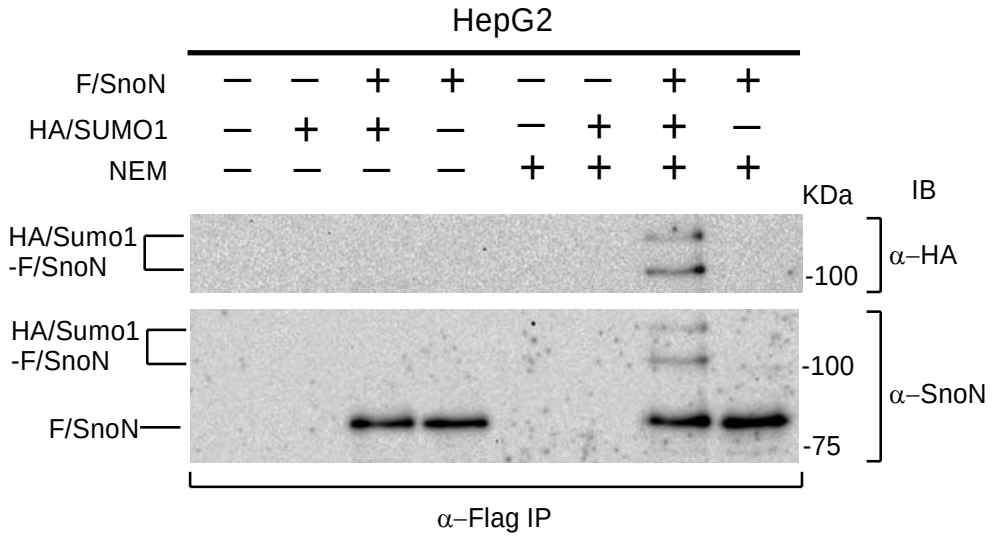
3.1.1 Overexpressed SnoN is modified by SUMO1

SnoN has positive and negative transcriptional regulatory functions. The mechanisms by which SnoN produces distinct transcriptional activities remain to be identified. Since sumoylation can impact the function of a transcription factor, it is possible that this modification may represent a mechanism by which SnoN transcriptional function is regulated. To test if SnoN can be conjugated by SUMO *in vivo*, FLAG-tagged SnoN either alone or together with HA-tagged SUMO1 were overexpressed in the human hepatoma HepG2 cells. Cell lysates prepared in the absence or presence of the general sumo-protease inhibitor N-ethylmaleimide (NEM) were immunoprecipitated with anti-FLAG antibodies followed by immunoblotting with antibodies to the HA tag. SUMO coexpression with SnoN in cells resulted in the appearance of NEM-sensitive slower mobility protein species in the SUMO blots of the SnoN immunoprecipitation (Figure 3-1A, compare lane 3 to 7). These SUMO immunoreactive protein bands were absent in the samples from cells expressing SnoN or SUMO alone (Figure 3-1A, lanes 2, 4, 6 and 8). Two reproducible NEM-sensitive SUMO-conjugated protein species displayed apparent molecular weights of ~100 and ~120 kDa differing from the 80 kDa SnoN protein by multiples of 20 kDa (Figure 3-1A, lane 7). As sumo-conjugated species of substrates have apparent molecular weight that differ by 20 kDa or greater from the unmodified substrates, the data suggest that the SUMO-conjugated protein bands in the SnoN immunocomplexes represents SUMO-conjugated forms of expressed SnoN. To

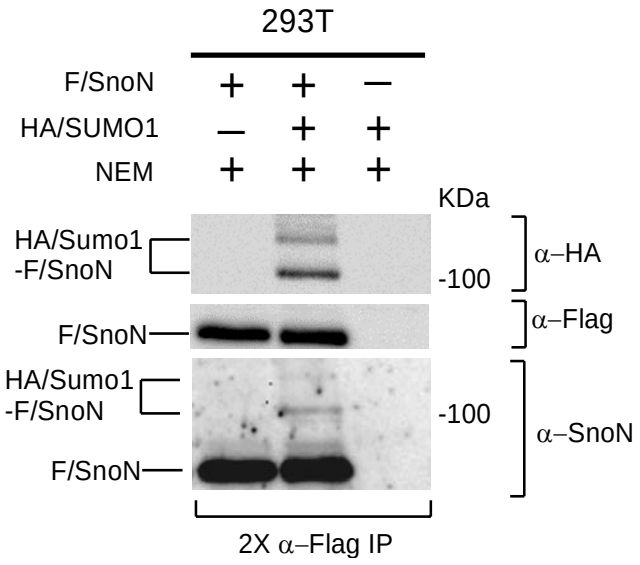
Figure 3-1 Expressed SnoN is modified by sumoylation

Expressed SnoN is sumoylated in mammalian cells. HepG2 (**A**) and 293T (**B**) were transfected with an empty mammalian expression vector (pCMV5B) or with Flag-tagged SnoN (Flag/SnoN) and HA-tagged SUMO (HA/SUMO)-expressing plasmids alone or together. Two days post-transfection cells were lysed in the presence (+) or absence (-) of the isopeptidase inhibitor N-ethyl maleimide (NEM) to preserve any sumoylated proteins. Lysates were subjected to a single α-Flag IP (**A**) or a double (2X) α-Flag IP (**B**) to precipitate expressed SnoN immunocomplexes. This was followed by immunoblotting (IB) with anti-HA, and anti-SnoN (**A**) or anti-Flag (**B**) antibodies, to visualize sumoylated protein species and SnoN, respectively, in the SnoN immunocomplexes. In addition, the 2X α-Flag IP blot was stripped and reprobed with anti-SnoN antibody to confirm that the HA-immunoreactive bands were also detected by anti-SnoN antibody (**B**).

A.


HepG2
F/SnoN
HA/SUMO1
NEM
KDa
IB
HA/Sumo1
-F/SnoN
-100
α–HA
HA/Sumo1
-F/SnoN
F/SnoN
-100
-75
α–SnoN
α–Flag IP

B.


293T
F/SnoN
HA/SUMO1
NEM
KDa
HA/Sumo1
-F/SnoN
-100
α–HA
F/SnoN
α–Flag
HA/Sumo1
-F/SnoN
F/SnoN
-100
α–SnoN
2X α–Flag IP

determine if SnoN is sumoylated in other cell types, we followed the appearance of SUMO-conjugated proteins in SnoN immunoprecipitates of lysates of the human kidney epithelial 293T transiently expressing SnoN, SUMO, alone or together. As was demonstrated in HepG2 cells, coexpression of SUMO and SnoN in 293T cells led to the appearance of the NEM-sensitive ~100 and ~120 kDa SUMO-immunoreactive protein species in SUMO-immunoblots of SnoN immunocomplexes (Figure 3-1B, lane 2 compared with lane 7 of 3-1A). In addition, these SUMO-conjugated protein species are present in SnoN immunoprecipitates of 293T lysates carried out under the denaturing conditions of the second FLAG IP (Figure 3-1B, see 2.5.2 for methods), thus excluding the possibility that SUMO-conjugated proteins in the SnoN immunoprecipitation are SUMO-conjugated species of SnoN-interacting proteins. Taken together, these data suggest that SnoN undergoes posttranslational modification by SUMO in different cell types.

3.1.2 Endogenous SnoN is modified by sumoylation

To determine if endogenous SnoN can be modified by SUMO, the presence of SUMO-conjugated endogenous SnoN were monitored in lysates of 293T cells transiently transfected with the HA/SUMO1 expression plasmid or its control vector. Expression of SUMO in these cells revealed two NEM-sensitive SUMO-conjugated SnoN protein species in endogenous SnoN immunoprecipitates of these cells. These results suggest that endogenous SnoN can be sumoylated in cells (Figure 3-2A, upper panel, compare lanes 1 and 2). Immunoblotting of the SnoN immunoprecipitates with the SnoN antibody revealed the presence of the slower migrating NEM-sensitive protein bands with similar apparent molecular weight as compared to the SUMO-conjugated SnoN detected in the

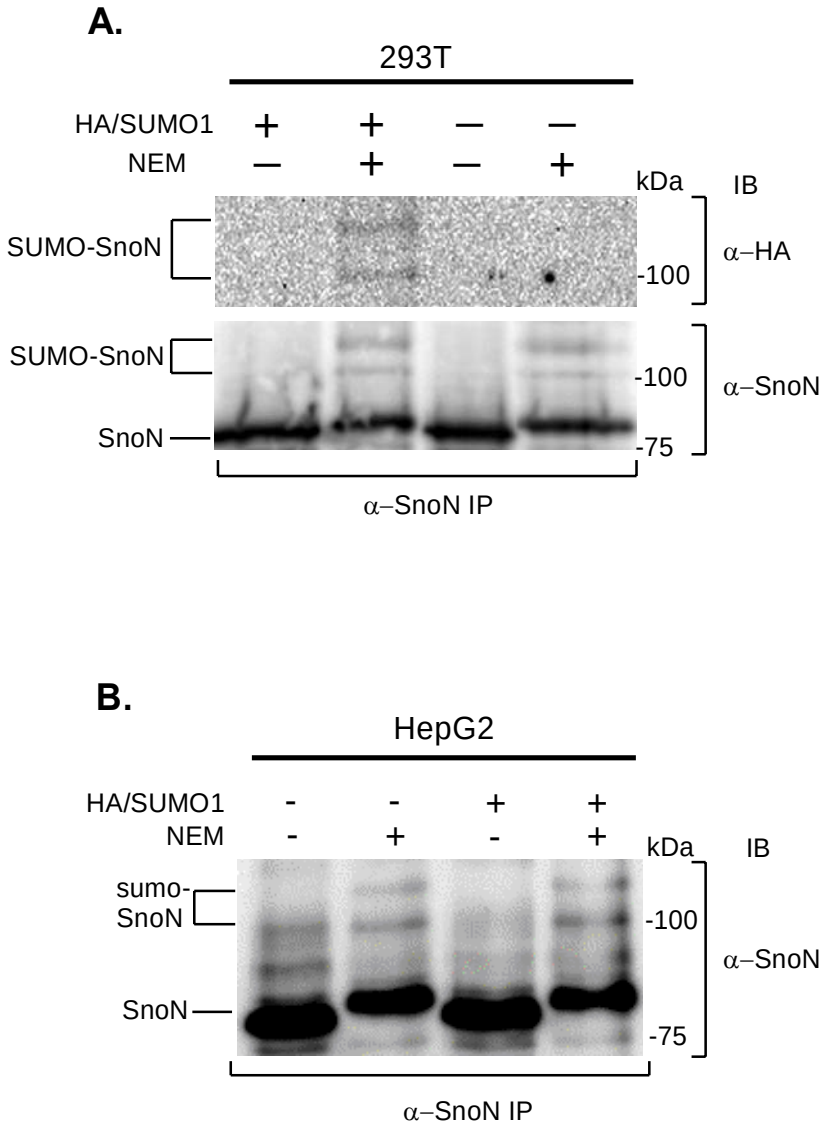


Figure 3-2 Endogenous SnoN is sumoylated

293T (A) and HepG2 (B) cells transfected with an empty pCMV5B or one encoding HA-SUMO1 were lysed in the presence (+) or absence (-) of NEM and the lysates were subjected to anti-SnoN immunoprecipitation followed by SUMO (anti-HA) and/or anti-SnoN immunoblotting.

HA blot (Figure 3-2A, refers to protein species labeled SUMO-SnoN in lanes 2 of upper and lower panels). Furthermore, SnoN immunoprecipitations of lysates of cells transfected with either the control or the SUMO expressing vectors displayed these SnoN-immunoreactive higher molecular weight species (Figure 3-2A, compare lanes 2 and 4 of lower panel), suggesting the possibility that endogenous SUMO content is sufficient to promote endogenous SnoN sumoylation in 293T cells. Results of similar experiments carried out in HepG2 cells further confirmed the presence of SUMO-conjugated SnoN protein bands in the immunoprecipitates of endogenous SnoN (Figure 3-2B). These results indicate that endogenous SnoN is a target of the SUMO pathway.

3.2 The SUMO pathway regulates SnoN sumoylation

Sumoylation of substrates is mediated by the SUMO conjugating pathway that consists of a cascade of three sets of enzymes (see 1.7.1). To demonstrate that the SUMO pathway controls SnoN sumoylation, the effect of the SUMO E2 conjugating enzyme Ubc9 on SnoN sumoylation was examined. SnoN sumoylation was assessed in NEM-treated lysates of 293T cells transiently coexpressing SUMO, SnoN, and a wild type or a catalytically inactive mutant Ubc9 protein in which cysteine 93 is mutated to a serine (Chakrabarti et al., 1999). Expression of wild type Ubc9 protein in cells coexpressing SnoN produced a robust increase in the total amount of SUMO-conjugated SnoN protein bands in SnoN immunocomplexes (Figure 3-3A compare lanes 2 and 4, and 3-3B). In contrast, expression of the catalytically inactive Ubc9 did not enhance but rather decreased SnoN sumoylation detected in the SnoN immunocomplexes (Figure 3-3A, compare lanes 2, 4 and 5) suggesting that this mutant of Ubc9 acts a dominant

negative to inhibit SnoN sumoylation by the endogenous Ubc9. Together the data demonstrate that the SUMO E2 enzyme Ubc9 is critical in the sumoylation of SnoN.

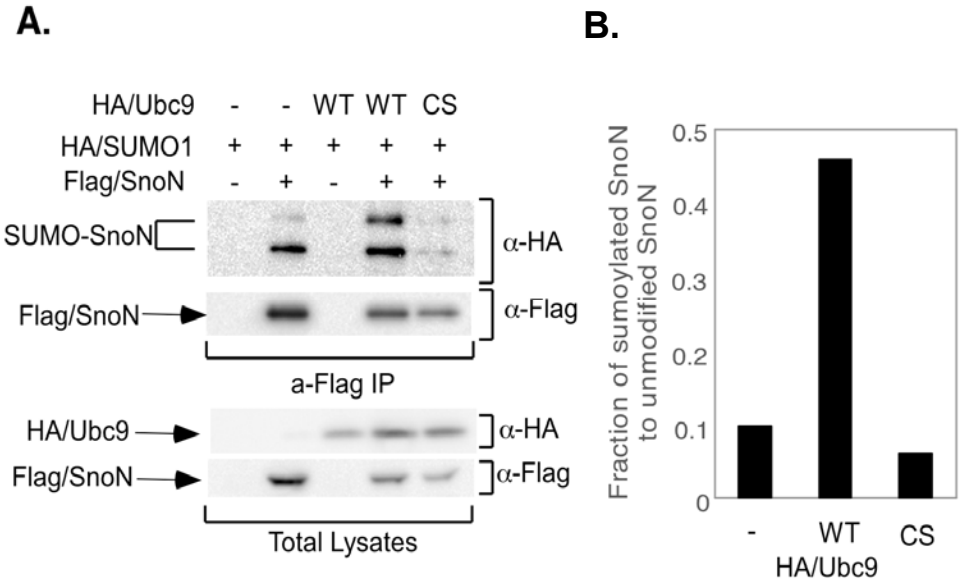


Figure 3-3 Ubc9 is critical for SnoN Sumoylation

A. SnoN sumoylation was determined in 293T cells transfected with Flag-tagged SnoN, HA-tagged SUMO, wild type or (C93S) mutant HA-tagged Ubc9 (HA/Ubc9). Expression of Flag/SnoN and HA/Ubc9 constructs in cell lysates were confirmed by immunoblotting with anti-Flag and anti-HA antibodies, respectively. **B.** Bar graph shows the density of total sumoylated SnoN normalized to the amount of unmodified SnoN in the SnoN-immunoprecipitation.

Next, the role of SUMO E3 ligases, which are believed to play a role in specifying substrates for sumoylation, in the modulation of SnoN sumoylation was investigated. Members of the PIAS family of SUMO E3 ligases associate with and promote the sumoylation of a number of transcriptional regulators (Muller et al., 2004). Therefore, an initial screen to determine if SnoN interacts with one or more members of the PIAS family of SUMO E3 ligases was performed. Coimmunoprecipitation studies of 293T cells expressing SnoN alone or together with PIAS1, PIAS3 or PIASy revealed that SnoN robustly forms a physical complex with PIAS1. In contrast, SnoN interacted weakly with other PIAS family members (Figure 3-4A, compare lane 4 to lanes 5 and 6). Quantitative analysis of PIAS-associated SnoN further confirmed that SnoN interacts in a selective manner with PIAS1 compared with other members of PIAS family of SUMO E3 ligases (Figure 3-4B). These data suggest that SnoN associates selectively with PIAS1.

The finding that SnoN interacts with the PIAS1 protein raised the possibility that SnoN sumoylation may be regulated by this SUMO ligase. Next, the effect of PIAS1 expression on SnoN sumoylation in 293T cells was examined. These experiments revealed that expression of PIAS1 together with SnoN enhances the sumoylation of SnoN (Figure 3-5A). The PIAS1-induced increase in SnoN sumoylation was confirmed by quantifying the density of total SUMO-conjugated bands relative to unmodified SnoN in the SnoN immunoprecipitates (Figure 3-5B). These data show that PIAS1 enhances SnoN sumoylation by more than 50%, indicating that PIAS1 acts as a SUMO ligase that regulates SnoN sumoylation. Taken together, these results suggest that PIAS1 represents a SUMO E3 ligase that stimulates the SUMO modification of SnoN.

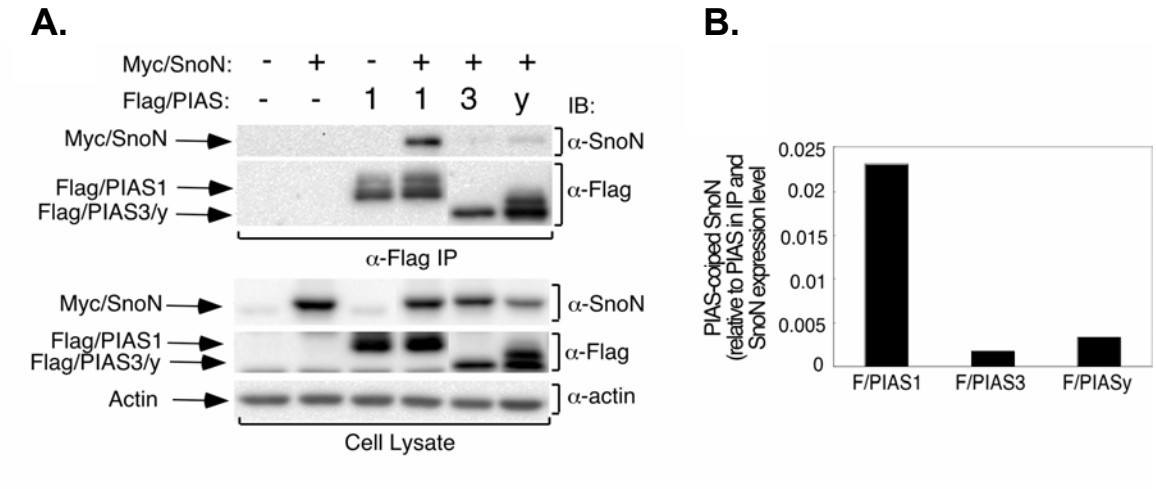


Figure 3-4 SnoN interacts selectively with the SUMO ligase PIAS1

A. NEM-treated lysates of 293T cells transiently expressing myc-tagged SnoN (myc/SnoN), alone or together with FLAG-tagged PIAS1, 3, or y were subjected to anti-FLAG IP and the PIAS-interacting SnoN protein was determined by anti-SnoN immunoblotting (IB). PIAS protein in the Flag-IP was confirmed by anti-Flag IB. Expression of SnoN and PIAS proteins was confirmed by Western blotting with SnoN and Flag antibodies, respectively. Actin was used as loading control. **B.** Bar graph shows the density of PIAS-interacting SnoN normalized to the amount of PIAS protein in the Flag-IP and Myc/SnoN expression in the respective transfection.

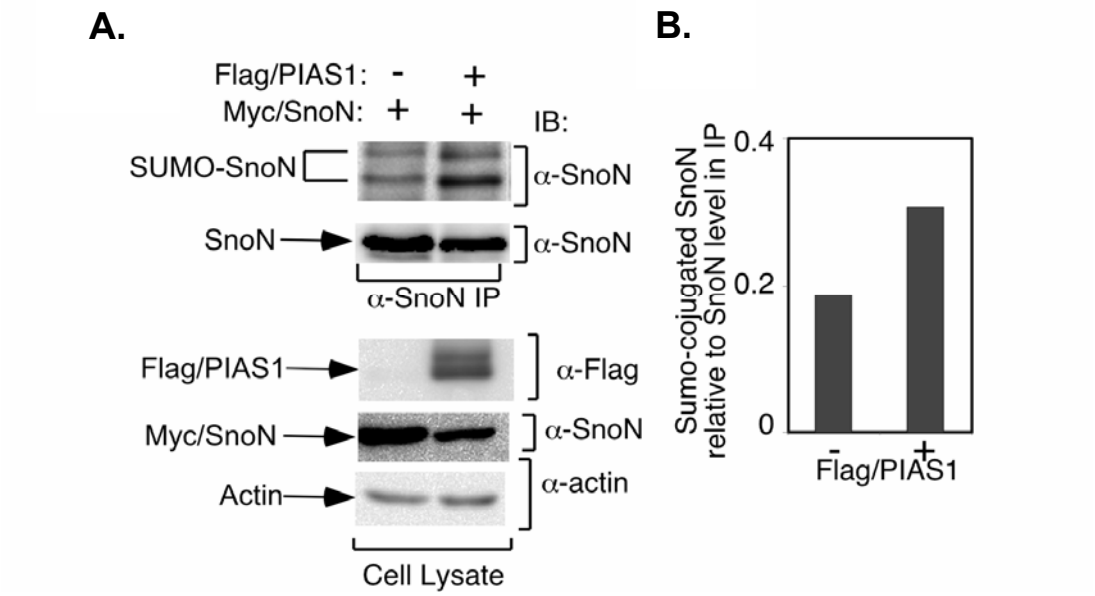


Figure 3-5 PIAS1 increases SnoN sumoylation

A. 293T cells were transfected with a pCMV5B plasmid containing Myc-tagged SnoN alone or together with a plasmid expressing FLAG-tagged PIAS1. SUMO-conjugated SnoN was determined by SnoN immunoblotting of SnoN-immunoprecipitation from NEM-containing lysates (upper panel). Expression of PIAS1, SnoN and actin proteins in cell lysates was confirmed as described in Figure 3-4A. **B.** Bar graph depicts the density of the two SUMO-SnoN protein bands normalized to the level of unmodified SnoN protein in the IP in the presence and absence of Flag/PIAS1.

3.3 Lysine residues 50 and 383 are the major sites of sumoylation on SnoN

Following the characterization of SnoN as a substrate for the SUMO pathway, I next sought to identify sites of sumoylation on SnoN. SUMO is conjugated to proteins via an isopeptide bond between the carboxyl group of the SUMO C-terminal glycine residue and the ε-amino group of a lysine residue in the substrate. Conjugation of SUMO moiety to proteins occurs on the substrate's lysine residues found within a ψKxE/D consensus motif, where ψ is a large hydrophobic residue and x is any amino acid (see 1.7.1). By sequence inspection, two SUMO consensus motifs were observed that are conserved in different species including human, mouse, and chicken SnoN (see Figure 1-5). To test if one or both lysine residues corresponding to residues 50 and 383 of human SnoN represent sites of sumoylation, expression vectors encoding human SnoN harbouring either lysine 50 (K50R), 383(K383R) or both residues (KdR) to arginine(s) mutation(s) were constructed (Figure 3-6A). Analysis of the effects of these mutations on SnoN sumoylation status in 293T cells revealed that mutation of lysine 50 of SnoN to arginine abrogated the 100 kDa SUMO-conjugated SnoN band, suggesting this protein represents lysine 50-sumoylated SnoN (Figure 3-6B, lane 2). By contrast, the level of a second lower mobility (~120 kDa) SUMO-conjugated SnoN protein species decreased drastically upon mutation of lysine 383 indicating that lysine 383-sumoylated SnoN represents the second SUMO-conjugated protein (Figure 3-6B, lane 3). The higher apparent molecular weight of the Lys383-sumoylated SnoN in SDS-PAGE begs the question if this site may be di-sumoylated. Since SUMO1 does not have a SUMO-conjugation consensus site, it is thought not to form SUMO chains, therefore

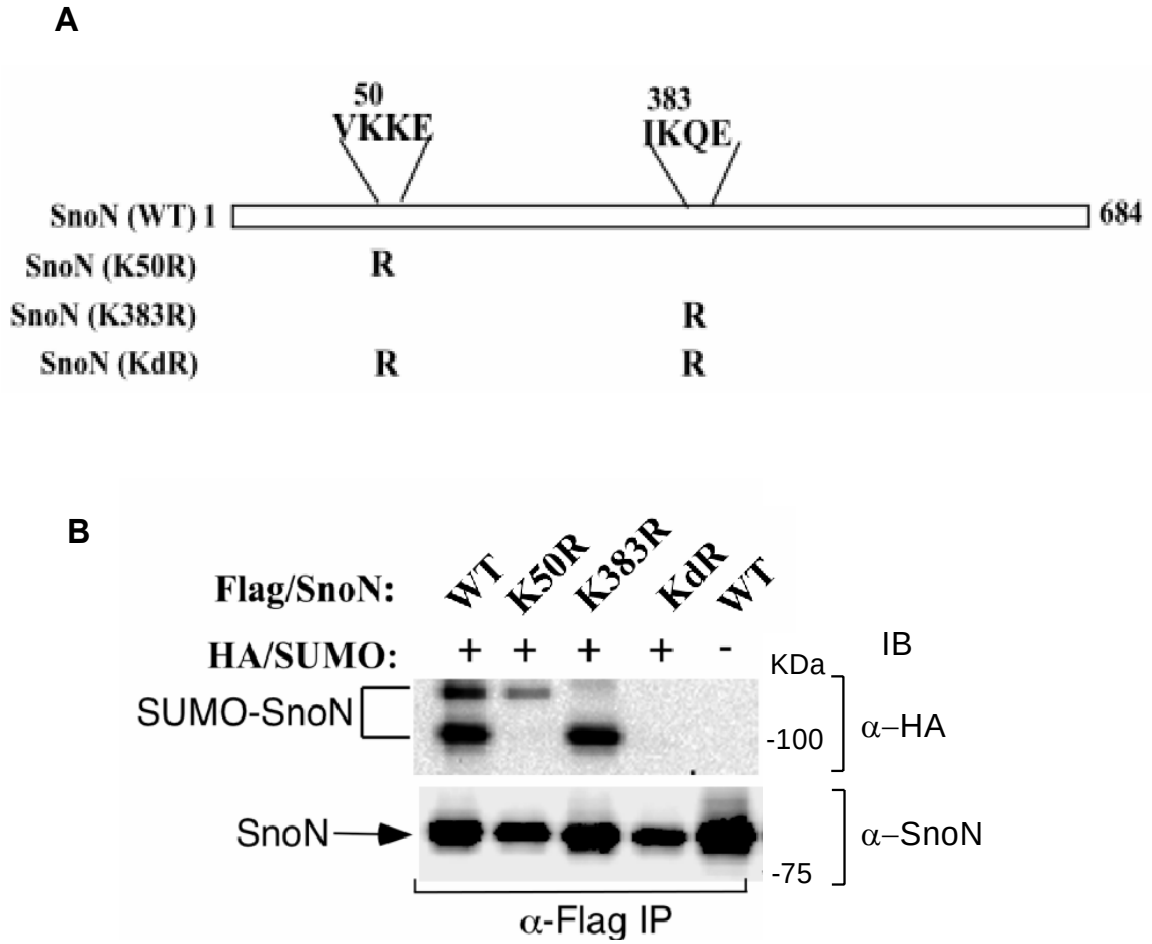


Figure 3-6 Lysine residues 50 and 383 are sites of sumoylation in SnoN

A. Schematic representation of the human SnoN protein with the lysine to arginine SnoN mutants generated in this study to determine potential sumoylation sites on SnoN. **B**. Identification of lysine sites of sumoylation in SnoN. Lysates of 293T cells coexpressing HA-tagged SUMO and Flag-tagged wild type SnoN (WT), or SnoN in which lysine 50 (K50R), lysine 383 (K383R) or both lysines (KdR) were mutated into arginine were subjected to anti-Flag IP followed by sequential anti-HA (upper panel) and anti-SnoN (lower panel) immunoblotting.

excluding the possibility that Lys383 is di-sumoylated. This electrophoretic migration anomaly, however, has been observed in other sumoylated proteins and has been suggested to likely result from the branching effect of the SUMO moiety (Perdomo et al., 2005; Kuo et al., 2005). As Lys383 resides approximately in the middle of SnoN which is a 684-amino acid protein, conjugation with a 100-amino acid SUMO at Lys383 would presumably create a branching arm, resulting in an aberrant migration on SDS-PAGE. Lys50, on the other hand, is near the N-terminus of SnoN. SUMO-conjugation at this site would create a more linear structure. As a result, Lys50-sumoylation increased the apparent molecular weight by the expected 20 kDa. Consistent with the above results that lysines 50 and 383 are sites of sumoylation, mutation of both lysine 50 and 383 led to the complete disappearance of both SUMO-conjugated protein bands in SnoN immunoprecipitates (Figure 3-6B, lane 4). Together, these data suggest that SnoN is sumoylated on lysines 50 and 383.

In summary, the data thus far indicate that SnoN is sumoylated and that lysines 50 and 383 are required for SnoN sumoylation. Moreover, SnoN sumoylation is strongly stimulated by E2 conjugating enzyme Ubc9 and E3 ligase PIAS1 of the SUMO pathway. In the sections below, the functional roles of SnoN sumoylation were investigated.

3.4 Sumoylation does not affect SnoN localization to the nucleus

Sumoylation has been shown to influence protein subcellular localization; in many cases, sumoylation results in retention of the substrate in the nucleus (Lin et al., 2003). Although SnoN generally localizes in the nucleus, cytoplasmic SnoN has also been detected (see Section 1.3). Therefore, it may be possible that sumoylation may influence SnoN subcellular localization. To test this idea, indirect immunofluorescence

experiments were carried out in HepG2 cells transiently expressing the wild-type or KdR versions of Myc-tagged SnoN protein to determine the subcellular localization of expressed SnoN (Figure 3-7). Cells were fixed and incubated first with mouse anti-Myc antibody and then with Cy™3-conjugated anti mouse antibody (excitation 550 nm, emission 570 nm) to detect ectopically expressed SnoN proteins. The nuclei were counterstained with DAPI. The colocalization of Cy™3 and DAPI signals suggested that both wild-type and KdR SnoN localize exclusively in the nucleus in HepG2 cells. These results thus indicate SnoN localization to the nucleus is not dependent on sumoylation. The experiments were repeated at least three times in HepG2 cells as well as Mv1Lu cells and yielded similar results (on average, 100 cells were counted per slide; data not shown).

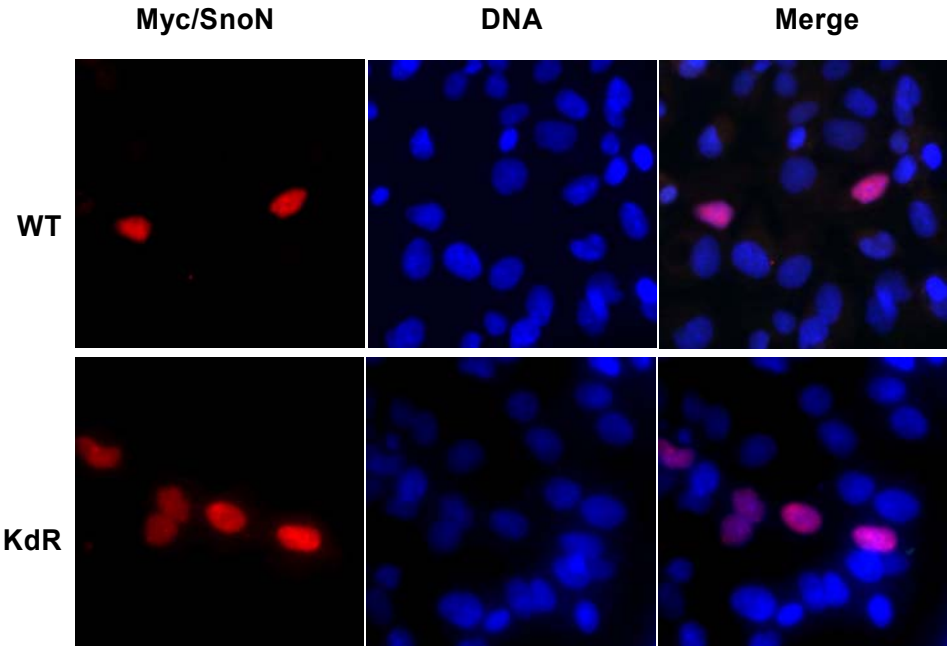


Figure 3-7 Sumoylation does not affect SnoN localization to the nucleus

HepG2 cells were transfected with a pCMV5B expression plasmid encoding Myc-tagged wild-type or KdR form of SnoN. Cells were fixed, permeabilized and stained for ectopically expressed SnoN with anti-Myc antibody (9E10) and Cy™3-conjugated anti-mouse secondary antibody. The nuclei were stained with 4'.6-diamidino-2-phenylindole (DAPI). The images were taken with a Zeiss Axiovert 200M fluorescent microscope equipped with an AxioCam MRc camera using 20X objective.

3.5 Sumoylation does not appear to alter SnoN protein stability

Sumoylation on the consensus lysine of its substrate has been suggested to increase the protein stability of its substrate. Another post-translation modification that utilizes lysine residues is ubiquitination which plays a role in protein degradation. It has been observed in some cases that the ubiquitin and SUMO can modify the same lysine residue. This observation has led to a model that SUMO-dependent regulation of protein stability occurs by excluding ubiquitin-modification on the same lysine site of protein. For example, NFκB inhibitor IκBα is ubiquitinated and sumoylated at the same lysine and the sumoylated species renders IκBα resistant to ubiquitin-proteasome degradation (Desterro et al., 1998). However, in the case of SnoN, sumoylation occurs on lysine residues 50 and 383, while ubiquitination occurs on lysine residues 440, 446 and 449 (Stroschein et al., 2001), suggesting that sumoylation is unlikely to regulate SnoN protein stability in this model. Alternatively, sumoylation may protect the substrate from degradation potentially by masking the lysine sites for ubiquitination. For example, a lysine to arginine mutation in the sumoylation site of Phosducin, a G-protein regulator, significantly lowers its protein stability as well as increases the ubiquitination compared with the wild-type Phosducin (Klenk et al., 2006), suggesting that the lysine residue in the sumoylation site was not a common site for ubiquitination and thus pointing to alternative mechanisms. Given this scenario, it was important to determine whether or not sumoylation plays a role in regulating SnoN protein stability. Two approaches, namely cycloheximide blocking and ^{35}S pulse-chase, were used.

3.5.1 Cycloheximide blocking analysis

Cycloheximide (CHX) is a eukaryotic protein synthesis inhibitor that is widely-used to investigate protein stability. Incubating cells with this reagent should inhibit new protein synthesis, thus allowing one to follow the decrease in the already synthesized proteins by degradation over time. To design these experiments, the amount of plasmid for transfection and the concentration of CHX to use for the experiments were first optimized. Various concentrations of CHX have been used in the literature. The concentrations of CHX ranging from 10 μg/ml to 80 μg/ml were tested in 293T cells. The concentration of CHX at 10 μg/ml has the least toxicity to 293T cells and is thus selected for the studies. Higher concentration of CHX (60 or 80 μg/ml) caused extensive cell death in 293 cells, thus affecting the results (data not shown). The level of expressed SnoN also required adjustment as a high level of expressed SnoN protein at time 0 resulted in a much extended half-life compared with the endogenous SnoN (data not shown). Therefore, the amount of SnoN protein was expressed at low level (50 ng/ml) in 293T cells to yield a more comparable half-life with the endogenous SnoN. Once the conditions were optimized, the stability of the wild-type (WT) SnoN or each of the SnoN sumo-mutants (K50R, K383R or KdR) was determined in 293T cells transiently expressing these proteins. The cells were treated with 10 μg/ml cycloheximide for the indicated time period and the level of SnoN protein at each time point was determined by Western blotting (Figure 3-8, upper panel). The overall rate of decrease was similar between the wild-type and the individual sumo-mutant of SnoN, indicating that the protein stability between each form of SnoN does not vary significantly (Figure 3-8,

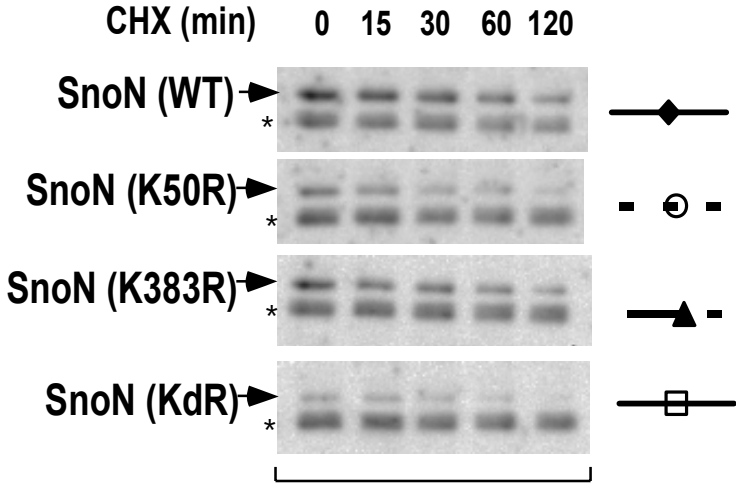
lower panel; shown as average of four independent experiments). These results suggest that sumoylation may not regulate SnoN protein stability.

3.5.2 ^{35}S pulse-chase analysis

To confirm the results the cycloheximide blocking analysis by another approach, ^{35}S pulse-chase analysis was carried out to assess the role of sumoylation on SnoN stability. The wild-type and sumo-mutant forms of SnoN transiently expressed in 293T cells were pulse-labelled with ^{35}S-methionine and chased for the indicated time period (see Section 2.6.2 for methods). In this method, SnoN half-life was determined for the newly synthesized SnoN proteins by monitoring the decrease in the intensity of radio-labelled SnoN over time. In the two experiments carried out, the overall decrease in SnoN levels were similar (Figure 3-9A & B; compare the end points between the different forms of SnoN). Interestingly, the stability of SnoN proteins appears higher in Figure 3-9A than in Figure 3-9B and both are higher than the SnoN protein stability determined by the cycloheximide blocking method. These observations suggest that the perceived stability of the protein may be influenced by different experimental methods (autoradiographic detection versus chemiluminescence) as well as inter-experimental variations (transfection efficiency). Taken together, sumoylation may not play a significant role in regulating the overall protein stability of SnoN. In summary, the results from both cycloheximide blocking and ^{35}S pulse-chase suggest that sumoylation does not appear to significantly affect the protein stability of SnoN.

Figure 3-8 Sumoylation does not affect the protein stability of SnoN determined by the cycloheximide blocking method

293T transfected with FLAG-tagged SnoN or its sumo-mutants were treated with 10 μg/ml cycloheximide (CHX) for the indicated time. Shown here is a representative blot in which the cell lysates were immunoblotted for SnoN with anti-FLAG antibody (upper panel). The arrow indicates the FLAG/SnoN band. The asterisks indicate a non-specific band. The immunoblots quantified and expressed as a percentage of the protein density prior to treatment were plotted in a line graph (lower panel). Each data point represents a mean (± SEM) from four independent experiments.


CHX (min)
0
15
30
60
120
SnoN (WT)
SnoN (K50R)
SnoN (K383R)
SnoN (KdR)
*
IB: α-FLAG
Total Lysate

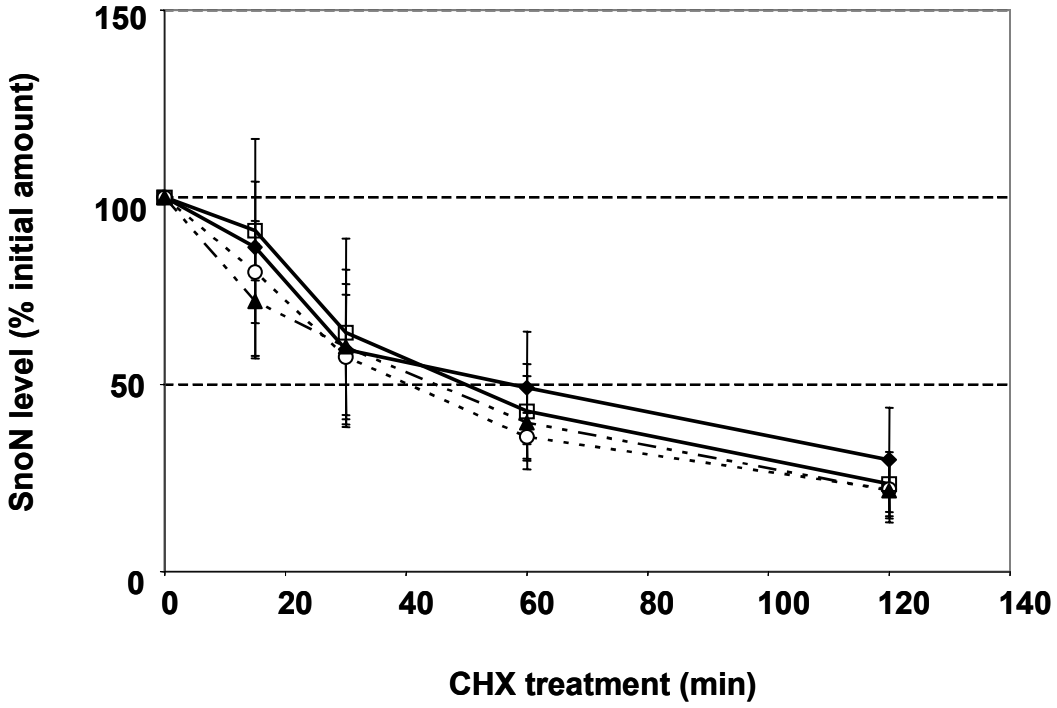
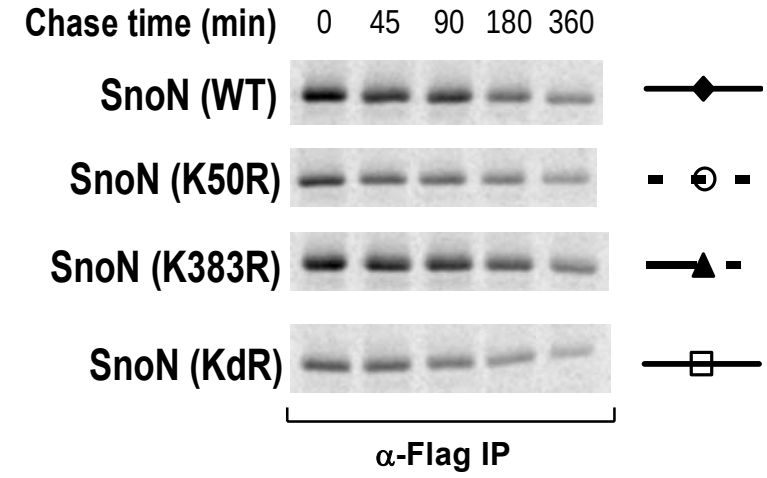
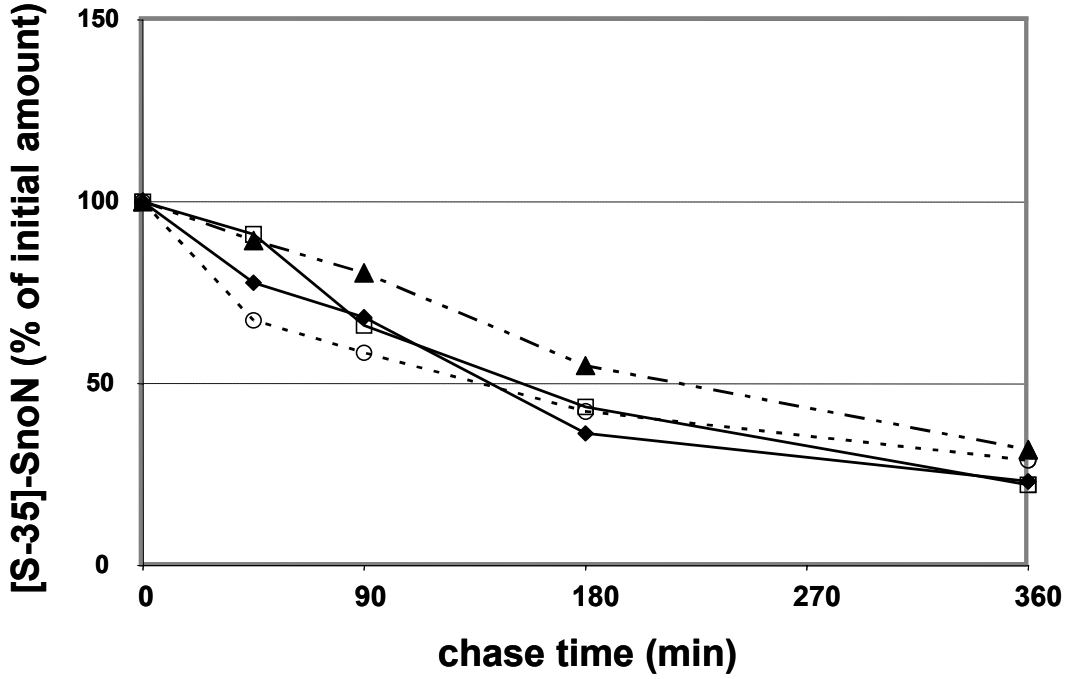

150
100
50
0
0
20
40
60
80
100
120
140
SnoN level (% initial amount)
CHX treatment (min)

Figure 3-9 Sumoylation does not affect the protein stability of SnoN determined by the ^{35}S pulse-chase method

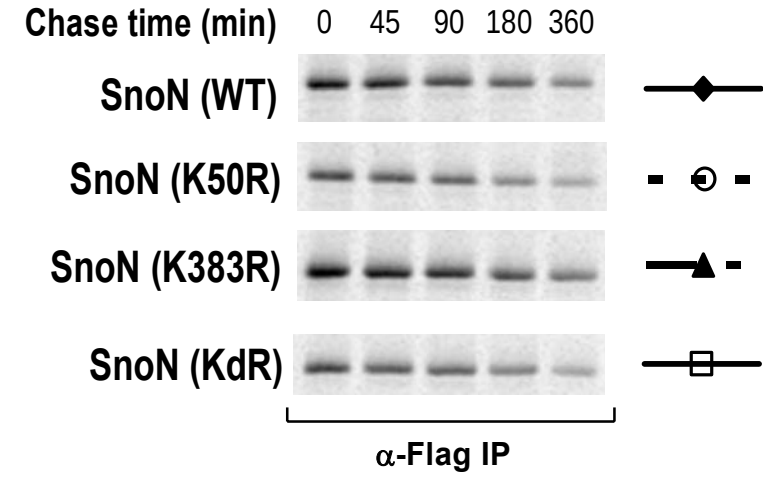
A & B. 293T transfected with FLAG-tagged wild-type and sumo mutants of SnoN were pulse-labelled with ^{35}S-methionine for 10 min and chased with regular DMEM containing nonradioactive methionine for the indicated time period. SnoN was immunoprecipitated with anti-FLAG IP and subjected to SDS-PAGE. The gel was fixed and dried, and then imaged by autoradiography (upper panels). The percent intensity of the labelled SnoN relative to time 0 was quantified by densitometry (lower panels).

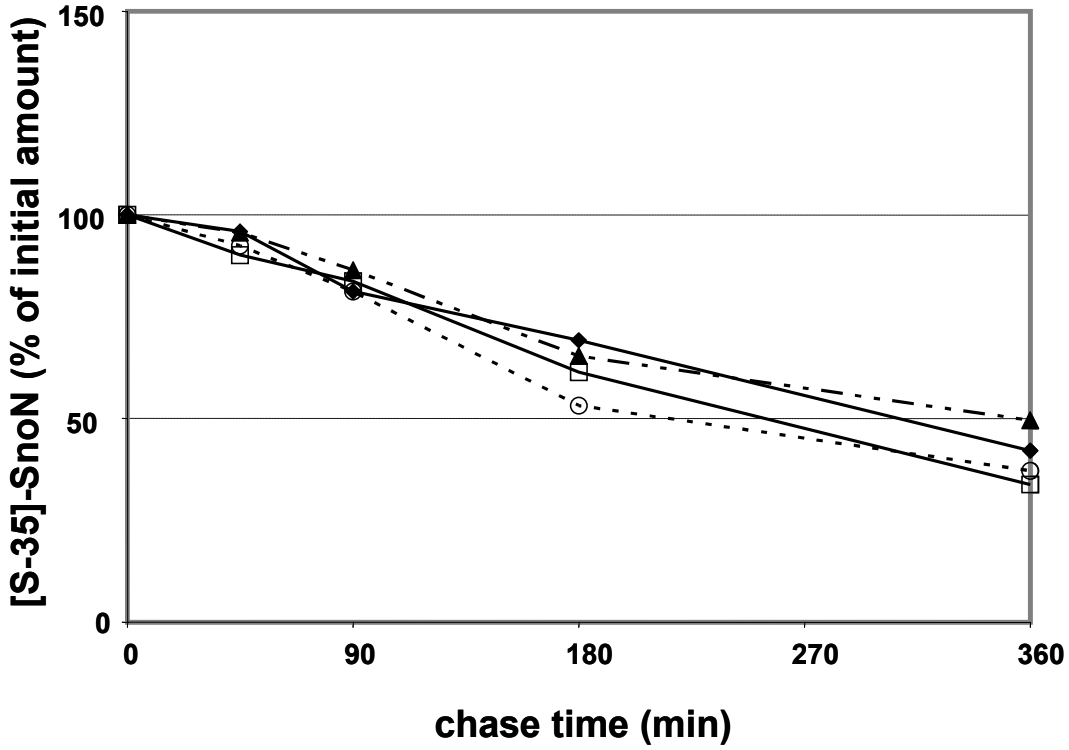
A.


Chase time (min) 0 45 90 180 360
SnoN (WT)
SnoN (K50R)
SnoN (K383R)
SnoN (KdR)
α-Flag IP


[S-35]-SnoN (% of initial amount)
150
100
50
0
0
90
180
270
360
chase time (min)

B.


Chase time (min)
0
45
90
180
360
SnoN (WT)
SnoN (K50R)
SnoN (K383R)
SnoN (KdR)
α-Flag IP

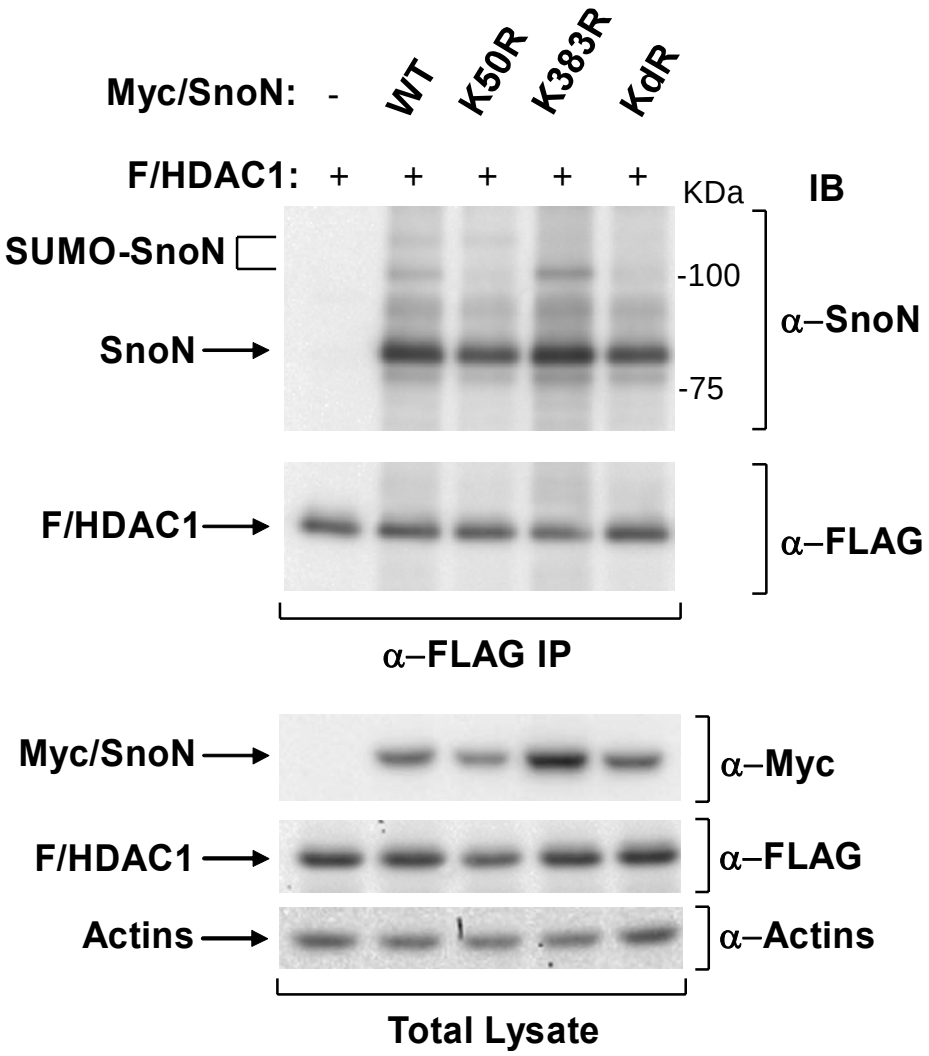

[S-35]-SnoN (% of initial amount)
150
100
50
0
0
90
180
270
360
chase time (min)

3.6 Sumoylation does not influence SnoN association with the HDAC complex

Sumoylation has been shown to positively or negatively regulate the association of transcriptional modulators with the HDAC complex. For instance, sumoylation of the transcription factor Ikaros leads to reduced association with the HDAC complex, while sumoylated p300 preferentially associates with HDAC6 (Girdwood et al., 2003; Gomez-del et al., 2005). SnoN has also been shown to recruit the HDAC complex to the TGF-β-regulated promoters to inhibit Smad-dependent transactivation of gene expression (Luo, 2004). Therefore, exploring the ability of wild-type SnoN and each of the SnoN sumo-mutants (K50R, K383R and KdR) to associate with HDAC could help explain a possible role of sumoylation of SnoN on the transcriptional activity of SnoN. To do this, coimmunoprecipitation studies of 293T cells expressing FLAG-tagged HDAC1 alone or together with the wild-type or sumo-mutant forms of Myc-tagged SnoN were carried out. As expected, SnoN (WT) was detected in the SnoN immunoblot of HDAC1 immunoprecipitates but was absent when HDAC1 was not coexpressed, indicating that SnoN associates with HDAC1 (Figure 3-10 lane 2 upper panel and data not shown). However, HDAC1 also interacts with K50R, K383R and KdR mutant of SnoN in a similar manner with the wild-type. These data suggest that sumoylation may not play a critical role in regulating SnoN association with the HDAC complex. In addition, based on the HDAC1 interaction with the wild-type SnoN, it appears that HDAC1 associates with both sumoylated and unmodified forms of SnoN proteins (Figure 3-10, lane 2 upper panel), pointing to the possibility that different HDAC complexes may exist *in vivo* that may preferentially contain sumoylated SnoN. Together, these results suggest that sumoylation may not regulate SnoN interaction with HDAC1.

Figure 3-10 Sumoylation does not affect the association of SnoN with HDAC1

NEM-treated lysates of 293T cells transiently expressing FLAG-tagged HDAC1 (F/HDAC1) alone or together with the wild-type or sumo-mutant (K50R, K383R or KdR) versions of Myc-tagged SnoN (Myc/SnoN) were subjected to anti-FLAG IP and the co-immunoprecipitated SnoN protein was determined by anti-SnoN immunoblotting. Expression of F/HDAC1 and Myc/SnoN constructs in cell lysates were confirmed by immunoblotting with anti-FLAG and anti-Myc antibodies, respectively. Actin was used as a loading control.


Myc/SnoN: - WT K50R K383R KdR
F/HDAC1: + + + + +
KDa
IB
SUMO-SnoN
SnoN
-100
-75
α–SnoN
F/HDAC1
α–FLAG
α–FLAG IP
Myc/SnoN
α–Myc
F/HDAC1
α–FLAG
Actins
α–Actins
Total Lysate

3.7 Regulation of SnoN sumoylation by TGF-β signalling pathway

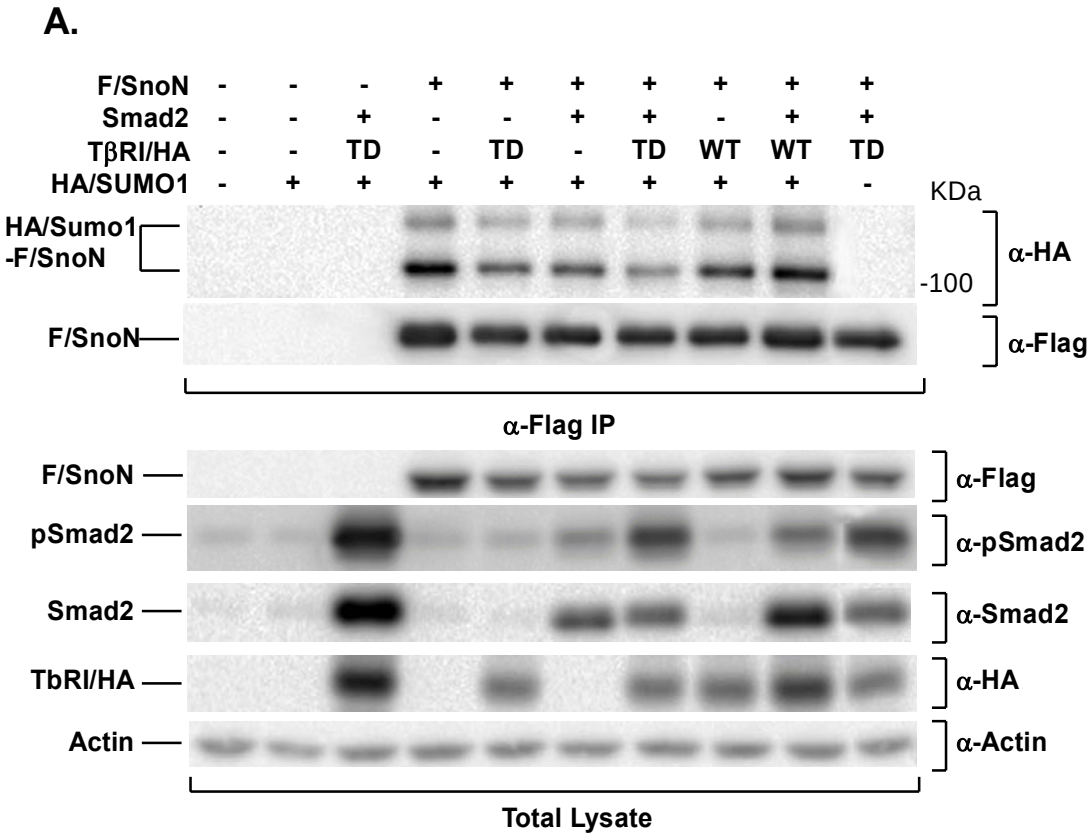
The TGF-β signalling pathway has been shown to regulate SnoN protein level (Wan et al., 2001; Stroschein et al., 2001; Bonni et al., 2001). SnoN is targeted for proteasomal degradation via TGF-β-dependent ubiquitination pathway, demonstrating that TGF-β signalling pathway may regulate a post-translational modification of SnoN. To investigate if SnoN sumoylation may also be controlled by the TGF-β signalling pathway, the level of sumoylated SnoN was determined in 293T cells transiently coexpressing SnoN and SUMO1 alone or together with the constitutively active mutant TGF-β type I receptor, TβRI (TD) in which Thr204 is mutated to Asp, or the wild-type receptor, TβRI (WT), and/or Smad2 (Figure 3-11A). The density of sumoylated SnoN species in the SnoN immunoprecipitates was consistently reduced by approximately 3 fold in the presence of activated receptor and Smad2 as compared to the IP from lysates of cells coexpressing SnoN and SUMO1 alone (Figure 3-11B). These results suggest that constitutive activation of TGF-β signalling may antagonize SnoN sumoylation.

In addition, it is important to determine if shorter time of TGF-β ligand stimulation can also regulate endogenous SnoN sumoylation. To investigate this, a time study was carried out in which TGF-β responsive HepG2 cells were incubated with 500 pM TGF-β for the different time intervals. Lysates of HepG2 cells treated at the indicated time intervals were subjected to SnoN immunoprecipitation analysis to determine the level of sumoylated SnoN (Figure 3-12). Surprisingly, the treatment of 500 pM TGF-β led to an increase in the level sumoylated SnoN species within the first 30 min (Figure 3-12B). Then, the relative level of sumoylated SnoN to unmodified SnoN decreased after 30 min of treatment to a similar level as before treatment (Figure 3-12B).

The level of the unmodified SnoN protein decreased within the first 30 min of treatment but was upregulated at 3 hours of treatment (Figure 3-12A). Due to the lack of a sensitive anti-SUMO1 antibody, the presumed sumoylated SnoN was determined by anti-SnoN immunoblotting based on the migration of the sumoylated SnoN bands as observed in Figure 3-2B. The transient increase in SnoN sumoylation by TGF-β was also observed in Mv1Lu cells stably expressing SnoN(WT), while these slower migrating sumoylated SnoN bands were absent in Mv1Lu cells stably expressing SnoN(KdR) (Dr. Stuart Netherton, personal communication). Taken together, these results suggest that SnoN sumoylation may be tightly controlled by the TGF-β signalling pathway, although the molecular mechanism and the significance of this requirement remain to be investigated.

Figure 3-11 Sumoylation of SnoN is regulated by the TGF-β signalling pathway

A. 293T cells transfected with FLAG-tagged SnoN and HA-tagged SUMO1, together with or without the wild-type TβRI (WT) or the constitutively activated TβRI (TD) and/or Smad2 were subjected to anti-FLAG IP. The presence of sumoylated SnoN was detected with anti-HA antibody in the SnoN immunoprecipitates. Expressions of transfected proteins were confirmed by immunoblotting. Phosphorylated Smad2 (pSmad2) was detected by phosphospecific anti-Smad2 antibody to indicate active TGF-β signalling. The blot shown is a representative of three independent experiments. B. Quantitation analysis to determine the level of sumoylated SnoN normalized to unmodified SnoN in the SnoN immunoprecipitates. The data points shown represent the mean (+SEM) of three independent experiments.


A.
F/SnoN - - - + + + + + + +
Smad2 - - + - - + + - + +
TβRI/HA - - TD - TD - TD WT WT TD
HA/SUMO1 - + + + + + + + + -
KDa
HA/Sumo1
-F/SnoN
-100
α-HA
F/SnoN
α-Flag
α-Flag IP
F/SnoN
α-Flag
pSmad2
α-pSmad2
Smad2
α-Smad2
TbRI/HA
α-HA
Actin
α-Actin
Total Lysate

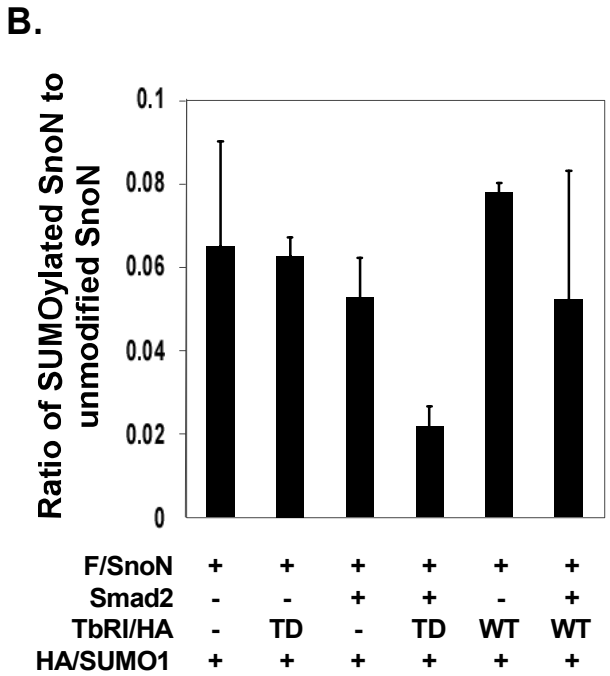
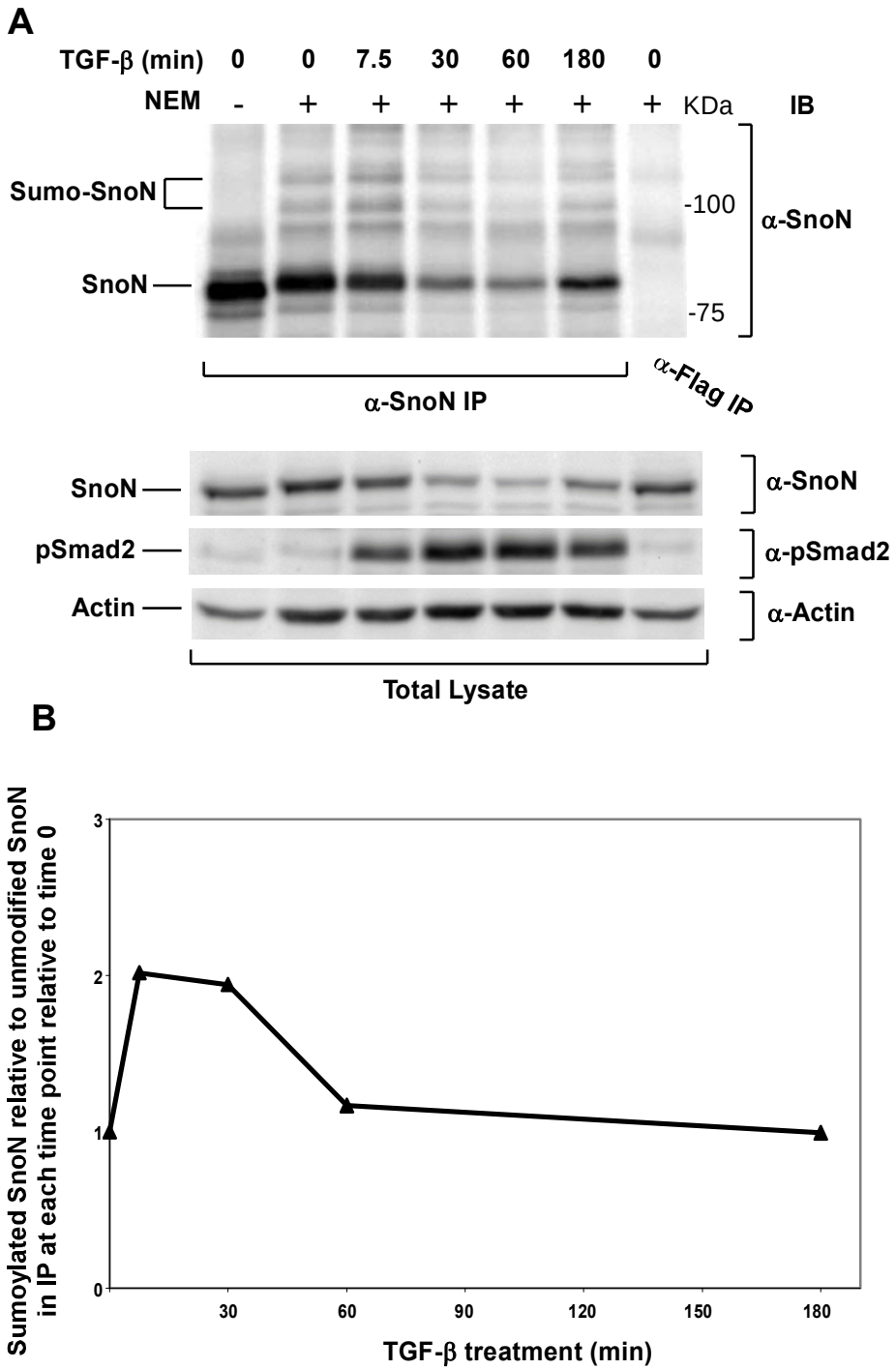

B.
Ratio of SUMOylated SnoN to unmodified SnoN
0.1
0.08
0.06
0.04
0.02
0
F/SnoN + + + + + +
Smad2 - - + + - +
TbRI/HA - TD - TD WT WT
HA/SUMO1 + + + + + +

Figure 3-12 TGF-β induces a transient increase in SnoN sumoylation

A. Endogenous SnoN in the lysates of HepG2 cells treated with 500 pM TGF-β for the indicated time period was immunoprecipitated, in the presence or absence of isopeptidase inhibitor, NEM as specified. The Anti-FLAG IP was used as a control IP. The anti-phospho-Smad2 (pSmad2) immunoblotting indicated active TGF-β signalling. **B.** Graph shows the ratio of sumoylated SnoN to unmodified SnoN in the SnoN IP at each time point relative to the time 0 control.


A
TGF-β (min) 0 0 7.5 30 60 180 0
NEM - + + + + + + KDa IB
Sumo-SnoN
SnoN
-100
-75
α-SnoN
α-SnoN IP
α-Flag IP
SnoN
pSmad2
Actin
α-SnoN
α-pSmad2
α-Actin
Total Lysate
B
Sumoylated SnoN relative to unmodified SnoN in IP at each time point relative to time 0
0
1
2
3
30
60
90
120
150
180
TGF-β treatment (min)

3.8 Role of Sumoylation on SnoN repression of TGF-β-dependent transcriptional activity

As shown in Section 3.7, SnoN sumoylation appeared to be affected by TGF-β signalling. When overexpressed, SnoN inhibits TGF-β-dependent transcriptional and biological responses (Luo, 2004). SnoN inhibition of TGF-β-dependent biological responses contributes to the role of SnoN as an oncogene (He et al., 2003). In view of these observations, the role of sumoylation in the ability of overexpressed SnoN to inhibit the TGF-β signalling pathway was investigated.

The luciferase reporter assay is widely used to study signal transduction and gene regulation. SBE4-luciferase reporter (SBE4-lux) is an artificial TGF-β-responsive reporter that is widely used in TGF-β signalling studies (Sun et al., 1999b; Ohshima and Shimotohno, 2003). SBE4-lux contains four repeats of Smad-binding element (SBE; GTCTAGAC) and its transcription is upregulated upon TGF-β stimulation (Sun et al., 1999b). Therefore, I decided to test the effect of SnoN sumoylation on TGF-β-dependent induction of SBE4-luc activity. To do this, HepG2 or Mv1Lu cells were transfected with an expression plasmid encoding SnoN (WT) or sumoylation-deficient mutant SnoN(KdR) together with SBE4-Lux and β-galactosidase expression plasmid, the latter serving as an internal control for transfection efficiency. Expression of SnoN (WT) in HepG2 and Mv1Lu strongly reduced the TGF-β-induced SBE4 luciferase reporter activity in a dose-dependent manner (Figure 3-13 and data not shown). However, the sumoylation-deficient mutant SnoN (KdR) was also found to repress the TGF-β-induced SBE4-lux activity in a similar fashion to the wild-type (Figure 3-13). The data suggest

that SnoN sumoylation has little effect on SnoN-mediated repression of TGF-β-dependent Smad transactivation.

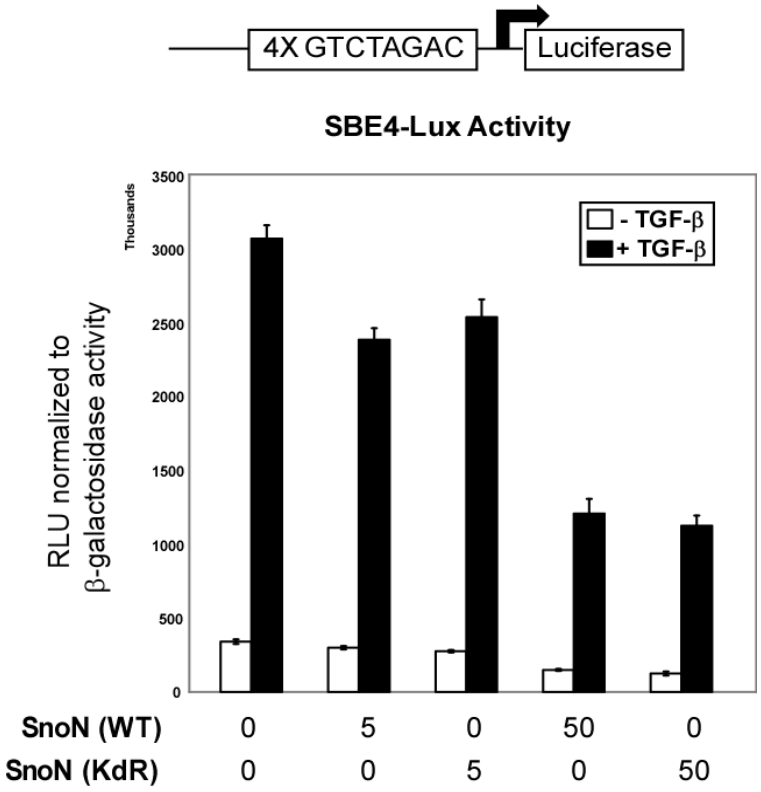


Figure 3-13 Sumoylation does not affect SnoN repression of SBE4-lux reporter activity

HepG2 cells transfected with two concentrations (ng/well) of SnoN(WT) or SnoN (KdR) together with SBE4-lux and β-galactosidase reporters were treated with or without 100 pM TGF-β for 20 h. Cell lysates were subjected to luciferase and β-galactosidase assays. The basal (- TGF-β) and TGF-β-induced (+ TGF-β) luciferase values are normalized to the β-galactosidase values (Relative Light Units, RLU). The results shown here represent the mean (+SEM) of a triplicate from a representative experiment that was repeated at least three times.

While in some cases synthetic reporter assays have been used to show changes in transcription activities of certain sumoylated transcription factors (Liang et al., 2004; Gomez-del et al., 2005), other transcription factors have demonstrated transcription regulations in a promoter-specific manner when sumoylated (Long et al., 2005; Perdomo et al., 2005; Wei et al., 2006). Therefore, it is possible that the sumoylation of SnoN may have a role in regulating natural TGF-β-responsive genes. To test this possibility, luciferase reporter assays were carried out to determine the effect of sumoylation on the ability of overexpressed SnoN to repress TGF-β-induction of the 3TP-lux reporter gene, which contains 3 repeats of AP-1 binding element (TRE) upstream of TGF-β-responsive elements from the promoter of the Plasminogen Activator Inhibitor-1 (PAI-1) gene driving the expression of the firefly luciferase gene (Wrana et al., 1992). HepG2 or mink lung epithelial MvLu cells were transfected with the 3TP-lux reporter gene together with an expression plasmid encoding wild type SnoN or the sumoylation-deficient mutant SnoN (KdR) together with a β-galactosidase expression plasmid. Expression of wild type SnoN decreased the basal activity and the TGF-β-induced 3TP-luciferase reporter gene in both HepG2 (Figure 3-14) and Mv1Lu cells (data not shown). SnoN (KdR) led to only 15 % reduction in SnoN-dependent repression of TGF-β-induced 3TP-lux activity in both HepG2 and Mv1Lu cells (Figure 3-14A and data not shown). In addition to using a "loss-of-function" approach of SnoN sumoylation in which sumoylation sites are mutated, a "gain-of-function" method was used to test if SnoN that is constitutively sumoylated is more potent in repressing TGF-β-dependent 3TP-lux activity in these cells. To do this, in collaboration with Dr. Isabelle Pot, a mammalian expression plasmid encoding a SnoN protein that is fused to the SUMO1 moiety in *cis* was constructed and

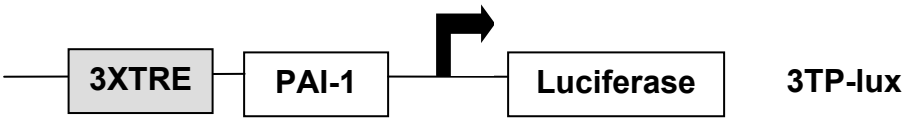
expressed in cells for 3TP-luciferase reporter assays. This approach has been successfully used to characterize the function of sumoylation of transcriptional regulators (Bossis et al., 2005; Long et al., 2004). In these experiments, SUMO-SnoN fusion protein only slightly increases the ability of SnoN to repress TGF-β-dependent 3TP-luciferase reporter gene expression in HepG2 and Mv1Lu cells (Figure 3-14B and data not shown). Together these data suggest that SnoN sumoylation has very little to no impact on the ability of overexpressed SnoN to inhibit TGF-β-induction of the PAI promoter.

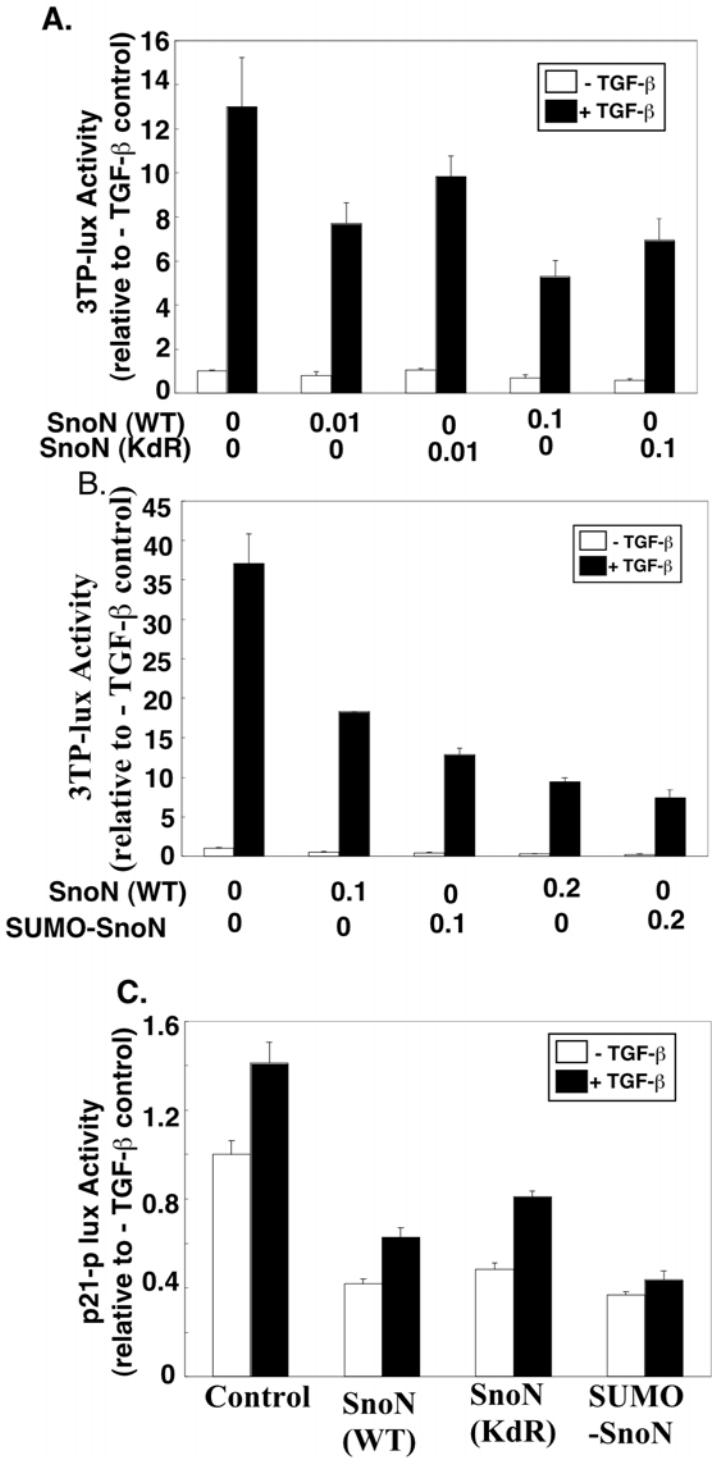
Next, the functional consequence of sumoylation of SnoN was determined on a TGF-β-responsive promoter distinct from the PAI promoter. The p21 cyclin-dependent kinase inhibitor gene is a TGF-β responsive gene that is important in TGF-β-inhibition of cell proliferation (Liu et al., 2001). A p21-lux reporter gene in which the luciferase reporter gene is controlled by a 2.3 kb fragment of the p21 promoter containing TGF-β-responsive elements was used (Beier et al., 1999). The effect of wild type SnoN, SnoN KdR, and the SUMO fusions of SnoN (SUMO-SnoN) on the response of the p21 promoter to TGF-β stimulation in HepG2 cells was compared in the p21-luciferase reporter assays (Figure 3-14C). Overexpression of wild type SnoN inhibited the basal and TGF-β-induced p21-promoter driven luciferase activity (Figure 3-14C). The SUMO-deficient KdR SnoN mutant was modestly less effective than wild type SnoN in repressing the p21 promoter gene. Conversely, SUMO-SnoN fusion protein repressed TGF-β-dependent p21 promoter activity more effectively than wild type SnoN (Figure 3-14C). These data suggest that sumoylation may potentiate the ability of overexpressed SnoN to suppress TGF-β-dependent p21 transcription. Together, these results suggest the

possibility that sumoylation of SnoN enhances the ability of SnoN to repress transcription in a promoter-specific manner.

Figure 3-14 Role of SnoN sumoylation on SnoN repression of TGF-β-induced transcription

SnoN sumoylation has a small or no effect on SnoN ability to repress the TGF-β induction of the 3TP-lux reporter activity (A & B). **A**. Lysates of HepG2 cells, transiently transfected with the 3TP-lux reporter and β-galactosidase constructs together with two concentrations (μg/well) of SnoN (WT) or SnoN (KdR), and either left untreated or treated for 20 h with 100 pM TGF-β were subjected to luciferase and β-galactosidase assays. The basal (- TGF-β) and TGF-β-induced (+ TGF-β) luciferase values are normalized to the β-galactosidase values (Relative Light Units, RLU). The normalized data are expressed relative to that of the control cells grown in the absence of TGF-β. **B.** Effect of SUMO "gain of function" SnoN protein on basal and TGF-β-dependent 3TP-lux activity. HepG2 cells were transfected with the 3TP-lux reporter and β-galactosidase constructs together with two concentrations of SnoN (WT) or a SUMO1-tagged SnoN (SUMO-SnoN) and processed as described in A. **C.** SnoN sumoylation modestly enhances SnoN repression of the TGF-β-responsive p21 promoter. HepG2 cells were transfected with the p21-p-lux reporter and β-galactosidase constructs together with SnoN (WT), SnoN (KdR), or SUMO-SnoN, and processed as described in A. The results shown in A, B and C represent the mean (+SEM) of a triplicate from a representative experiment that was repeated at least three times.


3XTRE
PAI-1
Luciferase
3TP-lux


A.
3TP-lux Activity
(relative to - TGF-β control)
- TGF-β
+ TGF-β
SnoN (WT) 0 0.01 0 0.1 0
SnoN (KdR) 0 0 0.01 0 0.1
B.
3TP-lux Activity
(relative to - TGF-β control)
- TGF-β
+ TGF-β
SnoN (WT) 0 0.1 0 0.2 0
SUMO-SnoN 0 0 0.1 0 0.2
C.
p21-p lux Activity
(relative to - TGF-β control)
- TGF-β
+ TGF-β
Control
SnoN (WT)
SnoN (KdR)
SUMO -SnoN

3.9 SnoN Sumoylation controls differentiation-induced myogenin promoter activity.

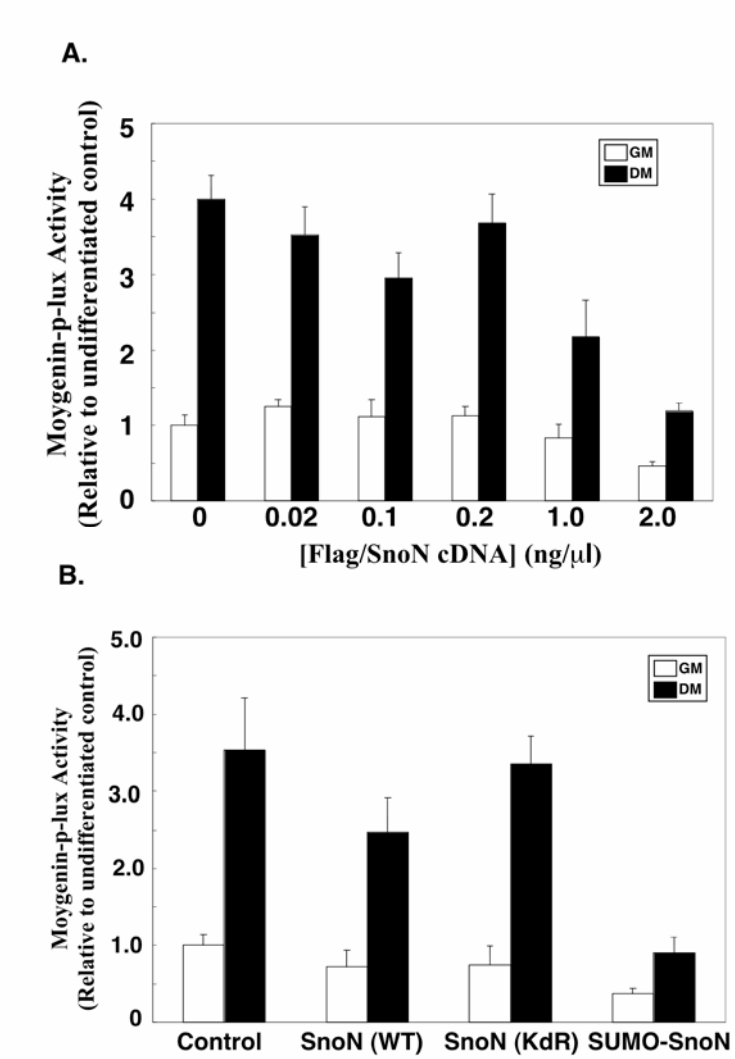
The finding that sumoylation of SnoN contributes to SnoN-dependent transcriptional repression in a promoter-specific manner led to the question of how SnoN sumoylation might contribute to SnoN function. Besides the control of cell proliferation, SnoN has been implicated in muscle differentiation. In collaboration with Dr. Krishna P. Sarker, the role of SnoN sumoylation in SnoN function in muscle differentiation was investigated. In these studies, mouse C2C12 myoblast cells were used as this cell line serves as a well characterized model for skeletal muscle differentiation. When grown in low serum containing medium, C2C12 cells express muscle markers and regulators including the transcription factor myogenin and adopt a skeletal muscle phenotype (Mimura et al., 1996). A luciferase reporter construct driven by 1.14 kb promoter of the mouse myogenin gene (myogenin-p-lux), a muscle master regulator gene, was used as a marker for muscle differentiation (Weston et al., 2003). The effect of increasing SnoN expression on the luciferase activity of the myogenin-p-lux reporter construct was first studied to assess a possible role of SnoN in regulating myogenesis. Luciferase and β-galactosdase assays were carried out on lysates of cells that were transiently transfected with the reporter and the SnoN expression constructs and either left in regular growth medium or switched to muscle differentiating medium (see Section 2.8.2 for details). Myogenin reporter transcription increases by 2 to 4 fold when cells are induced to undergo muscle differentiation (Figure 3-15A). At lower concentrations, SnoN produced little change in the myogenin-promoter driven luciferase activity. However at higher doses, SnoN appeared to inhibit differentiation-induced myogenin promoter

activity by 75%. These data show that SnoN inhibits muscle-dependent activation of the myogenin promoter in a dose-dependent manner.

Next, to address if SnoN sumoylation plays a role in the ability of SnoN to inhibit the myogenin promoter activity, luciferase reporter experiments were carried out to investigate the effects of the SUMO loss of function and gain of function SnoN mutants on the myogenin-p-lux transcriptional activity in undifferentiated or muscle-differentiated C2C12 cells. In these experiments, a concentration of wild type SnoN producing submaximal inhibition of the myogenin promoter was used (see Figure 3-15A). In contrast to wild type SnoN which inhibited the myogenin promoter, the sumoylation-deficient mutant SnoN (KdR) did not significantly reduce the activity of the myogenin promoter, suggesting that sumoylation of SnoN at lysine 50 and 383 might contribute to the ability of SnoN to inhibit myogenin promoter activity (Figure 3-15B). In agreement with this interpretation, the gain of function SUMO-SnoN protein robustly inhibited the luciferase activity as compared to the wild type SnoN control (Figure 3-15B). Therefore these data suggest that sumoylation of SnoN enhances the ability of SnoN to repress the myogenin promoter activity. Taken together, the results of the transcriptional assays support the idea that sumoylation may provide a mechanism whereby SnoN may impact repression of specific promoters.

Figure 3-15 Sumoylation of SnoN promotes SnoN transcriptional repression of the muscle-specific myogenin promoter activity

A. SnoN inhibits the myogenin promoter activity in a concentration dependent manner. C2C12 cells were transfected with the myogenin-p-lux reporter and β-galactosidase constructs together with an empty pCMV5B plasmid or increasing concentrations of pCMV5B plasmid encoding Flag-tagged SnoN protein. Confluent transfected cells were kept in regular growth media (GM) or switched to muscle differentiating media (DM). Two days later, cells were lysed and subjected to luciferase and β-galactosidase assays. The basal (GM) and differentiation-induced (DM) myogenin driven luciferase values were normalized to the β-galactosidase values (Relative Light Units, RLU) and expressed relative to the normalized luciferase activity in lysates of control cells grown in regular media (GM). **B.** SnoN sumoylation promotes SnoN repression of the myogenin promoter. C2C12 cells were transfected with the myogenin-p-lux reporter and β-galactosidase constructs together with pCMV5B plasmid encoding wild type (WT) SnoN, a SUMO "loss of function" SnoN (KdR) mutant or the SUMO "gain of function" SUMO-SnoN mutant. Cells were processed as described in A. The results shown in each of A and B represent the mean (+SEM) of a triplicate from a representative experiment that was repeated at least three times.


A.
Moygenin-p-lux Activity
(Relative to undifferentiated control)
5
4
3
2
1
0
GM
DM
0
0.02
0.1
0.2
1.0
2.0
[Flag/SnoN cDNA] (ng/μl)
B.
Moygenin-p-lux Activity
(Relative to undifferentiated control)
5.0
4.0
3.0
2.0
1.0
0
GM
DM
Control
SnoN (WT)
SnoN (KdR)
SUMO-SnoN

Chapter Four: Discussion and Future Directions

4.1 SnoN sumoylation

In this study, SnoN was identified as a target of sumoylation. The covalent modification of SnoN by SUMO was observed in distinct cell types. This indicates that SnoN sumoylation may be a widely-used post-translational modification for controlling SnoN functions. Mutation analysis mapped the sumoylation sites to lysines 50 and 383 (Figure 3-6), which correspond to the two conserved sumoylation consensus motifs found in SnoN (Figure 1-5). The different electrophoretic mobilities of K50- and K383-sumoylated SnoN proteins in SDS-PAGE revealed the existence of two distinct pools of sumoylated SnoN species, allowing assessment of the relative abundance of the two sumoylated species. The K50-sumoylated SnoN appears to be the more abundant sumoylated form than the K383-sumoylated species, particularly in 293T cells (Figure 3-1B). The functional significance of the individual sumoylation site, however, is presently undefined. Interestingly, Lys50 resides in the previously identified repression subdomain spanning the first 85 amino acid residues (Cohen et al., 1998). The SUMO consensus motifs are often located within repression domains of transcription regulators, indicating the possible involvement of sumoylation in gene-silencing (Muller et al., 2004). This may suggest a role for Lys50-sumoylation of SnoN in transcriptional repression. Evidence for any structural relation of Lys383 in SnoN is very limited.

Sumoylation occurs via a 3-step enzymatic cascade (see Section 1.7.1). The SUMO E2 conjugating enzyme Ubc9 binds directly to the sumo consensus motif in the substrate. A SUMO E3 ligase assists in specifying the substrate, and in cooperation with

Ubc9, facilitates the transfer of the SUMO moiety from the active site cysteine in Ubc9 to the lysine residue in the consensus motif of the substrate. In this study, SnoN sumoylation was found to be strongly stimulated by overexpression of Ubc9. In addition, SnoN interacts selectively with PIAS1 (Figure 3-4), a known SUMO E3 ligase for a number of transcription factors such as Sp3 and Smad4 (Sapetschnig et al., 2002; Liang et al., 2004). Furthermore, expression of PIAS1 consistently enhanced SnoN sumoylation *in vivo* supporting the idea that PIAS1 is the SUMO E3 for SnoN sumoylation (Figure 3-5). Together, these data suggest that components of the SUMO pathway may play a role in controlling the level of SnoN sumoylation, which in turn may impact SnoN functions in various cellular processes.

The consequences of sumoylation on the function of transcriptional factors and coregulators occur via distinct mechanisms (Johnson, 2004). In some cases, sumoylation regulates the function of substrates via changes in substrate protein subcellular localization (Lin et al., 2003). The potential role of SnoN sumoylation in regulating SnoN subcellular localization was examined in this study. However, double sumoylation-deficient mutant SnoN (KdR) localized to the nucleus to a similar extent as the wild-type SnoN (Figure 3-7). This suggests that SnoN sumoylation does not play an essential role in the subcellular distribution of SnoN.

The finding that PIAS1 specifically interacts with and enhances the sumoylation of SnoN may add another angle to the functional study of SnoN sumoylation in regulating protein subnuclear localization that has not been explored in this project. PIAS family proteins can associate with nuclear matrix through their SAP domain (Shuai and Liu, 2005). In addition, PIAS proteins have been shown to target their substrates to

subnuclear structures such as nuclear bodies (Sachdev et al., 2001). SnoN interaction with PIAS1 may potentially lead to PIAS1-mediated subnuclear targeting of SnoN to distinct subnuclear compartments. Therefore, future studies aimed at determining whether or not PIAS1 can target SnoN to distinct nuclear bodies should reveal interesting insights. Additionally, if PIAS1 is found to target SnoN to distinct subnuclear sites, it will be important to determine whether or not the PIAS1-mediated relocalization of SnoN requires the presence of the SnoN sumoylation sites.

In addition to regulating substrate localization, sumoylation has also been shown to regulate the protein stability of the substrate (Johnson, 2004). Therefore, the effect of SnoN sumoylation on the protein stability of SnoN was investigated in this study. As determined by cycloheximide blocking and ^{35}S-pulse chase experiments, no appreciable differences were observed between SnoN (WT) and the individual sumo-mutants (K50R, K383R and KdR) (Figure 3-8 and 3-9). However, a potential caveat in comparing the wild-type and sumo-mutants is that the majority of expressed SnoN is unsumoylated. The current method did not compare directly the half-life of sumoylated SnoN with that of unsumoylated SnoN. Furthermore, only the role of SnoN sumoylation on SnoN protein stability under the normal condition has been addressed in this study. As SnoN has been shown to be targeted for ubiquitin-proteasome degradation upon TGF-β stimulation, future studies should focus on the role of sumoylation in TGF-β-dependent SnoN degradation.

Since the TGF-β/Smad2 signalling pathway has been shown to regulate SnoN ubiquitination, sumoylation of SnoN may potentially be another modification targeted by this cytokine. The level of SnoN sumoylation in 293T cells was found to be consistently

reduced in the presence of constitutively active TGF-β signalling produced by the coexpression of activated TGF-β type I receptor TβRI(TD) and Smad2 (Figure 3-11), indicating that the TGF-β signalling pathway antagonizes SnoN sumoylation. However, using a different approach, in which the TGF-β-responsive HepG2 cells were used, SnoN sumoylation appears to increase transiently within the first 30 minutes of TGF-β-ligand stimulation and return to similar level as in the basal state in 3 hours (Figure 3-12). The TGF-β signalling pathway seems to be involved in SnoN sumoylation, although the exact mechanism and functional implication of TGF-β signalling in SnoN sumoylation are not clear. It is possible that TGF-β has cell type-specific effects in regulating the level of SnoN sumoylation. It should also be noted that results from these experiments cannot exclude the possibility that TGF-β may regulate the distribution of SnoN sumoylation between nucleoplasmic soluble fraction and the insoluble fraction instead of the level of SnoN sumoylation. The method used for processing cell lysate in this study only allowed for the analysis of the cytonucleoplasmic-soluble fraction of sumoylated SnoN. Consequently, SnoN sumoylation observed by Western blotting only revealed the changes in the pool of soluble fraction of SnoN and its sumoylated forms. Therefore, understanding the mechanism by which TGF-β signalling regulates SnoN sumoylation will be an important area of future investigations.

4.2 Sumoylation as a regulator of SnoN transcriptional repression

Growing evidences indicate that the majority of SUMO substrates are transcriptional factors and coregulators that activate or repress gene expression (Muller et al., 2004). Although sumoylation of some transcriptional factors including the β-catenin activated transcription factor TCF-4, and the heat shock factors HSF1 and HSF2 appear

to increase the transcriptional activity of target substrates (Yamamoto et al., 2003; Hong et al., 2001; Goodson et al., 2001), in most cases SUMO modification favors transcriptional repression by the substrates (Muller et al., 2004). Surprisingly, sumoylation enhances significantly the ability of SnoN to repress myogenin promoter-driven reporter gene activity (Figure 3-15). In contrast, SnoN sumoylation has a modest effect on the TGF-β-responsive p21-promoter activity and has little or no effect on the TGF-β-responsive PAI-1 driven 3TP-lux luciferase reporter gene and the artificial TGF-β-responsive SBE4-lux reporter (Figure 3-13 and 3-14). In the future, it will be important to determine the effect of SnoN sumoylation on the mRNA expression of these genes by quantitative reverse transcriptase PCR.

The data in this study support the idea that SnoN sumoylation plays a role in the ability of SnoN to repress transcription in a promoter-specific manner. This conclusion is supported by several recent studies focusing on the consequence of sumoylation on the function of different transcriptional regulators (Long et al., 2005; Perdomo et al., 2005; Wei et al., 2006). For example, sumoylation was found to potentiate the ability of the basic Krüppel-like factor BKLF to repress a glucocorticoid responsive element whereas this modification did not affect BKLF-dependent inhibition of the γ-globin promoter activity (Perdomo et al., 2005). Similarly, sumoylation of the transcriptional factor SIP1 was found to relieve SIP1-mediated repression of the E-cadherin promoter activity while having no effect on the ability of SIP1 to repress TGF-β-responsive elements or activate vitamin D3-responsive elements driven reporter genes (Long et al., 2005). It will be interesting in future studies to investigate the extent by which sumoylation contributes to the ability of SnoN to repress the activity of promoters of other SnoN-regulated genes.

Sumoylation has emerged as a key modification in regulating transcriptional repression. Currently, two models of mechanism by which SUMO-modification potentiates transcriptional repression have been proposed. These two models are not mutually exclusive and may act in concert with each other. One mechanism suggests that SUMO-modified proteins acquire the ability to recruit the HDAC complex to promoters (Girdwood et al., 2003; Yang and Sharrocks, 2004). The other mechanism proposes that SUMO-modification may target its substrate to subnuclear structures where the substrate can interact with the resident corepressors and become integrated into a transcriptional corepressor complex for gene-silencing (Sapetschnig et al., 2002). In this study, sumoylation of SnoN was not found to play a role in SnoN interaction with HDAC1 (Figure 3-10). However, HDAC1 appears to interact with both unmodified SnoN and sumoylated SnoN, suggesting the existence of different SnoN-HDAC complexes *in vivo* (Figure 3-10). This seems likely to explain the observation that SnoN specifically represses the *myogenin* promoter, as it is possible that SnoN sumoylation is essential for SnoN association with the corepressor complex in a promoter-specific manner that mediates the transcriptional repression of Myogenin. Sumoylation-deficient KdR SnoN, on the other hand, may be excluded from such complex, limiting its effect on Myogenin repression. Thus, SUMO-SnoN fusion protein represents a gain of function of mutation such that the noncleavable SUMO moiety renders the SnoN more efficient in associating with the corepressor complex to repress the Myogenin promoter. As differential targeting of sumoylated proteins to certain promoters has been observed (Kuo et al., 2005), it will be interesting to determine whether or not SnoN also exhibits this property by electrophoretic mobility shift assays and chromatin immunoprecipitation assays.

Also, it will be important to explore and characterize different corepressor complex with which SnoN may associate.

The finding that sumoylation can potentiate SnoN-dependent repression of the transcriptional activity of the myogenin promoter indicates that SUMO-conjugated SnoN may negatively regulate muscle differentiation. In the early studies, high level of SnoN was found to induce myogenic differentiation in quail embryo cells (Boyer et al., 1993). However, it has been suggested that this ability of SnoN may be characteristic of only avian species (Pearson-White, 1993). Nevertheless, the presence of elevated level of SnoN in embryo and neonatal skeletal muscles points to a role of SnoN in regulating myogenesis (Pearson-White, 1993; Pelzer et al., 1996; Pearson-White and Crittenden, 1997). It may be possible, as suggested in this study, that SnoN sumoylation may regulate at least one myogenic regulatory factor to control muscle differentiation. Under this scenario, expression of KdR sumo-deficient SnoN mutant would resemble a drastic reduction in the sumoylation of SnoN, hence promoting myoblast differentiation into skeletal muscle. Conversely, expression of the SUMO-SnoN fusion protein should act similarly to an increase in sumoylated SnoN, thus blocking or reducing the muscle differentiation program in myoblast cells. Therefore, this study presents a potential model for a functional role of SnoN sumoylation in muscle differentiation (Figure 4-1). Future studies should be directed at testing this model.

In summary, this thesis project presents the first finding that SnoN is post-translationally modified by SUMO1 at lysines 50 and 383. This modification enhances the ability of SnoN to suppress transcription of specific genes. These findings suggest that sumoylation may contribute to the functional diversity of SnoN in distinct cell types.

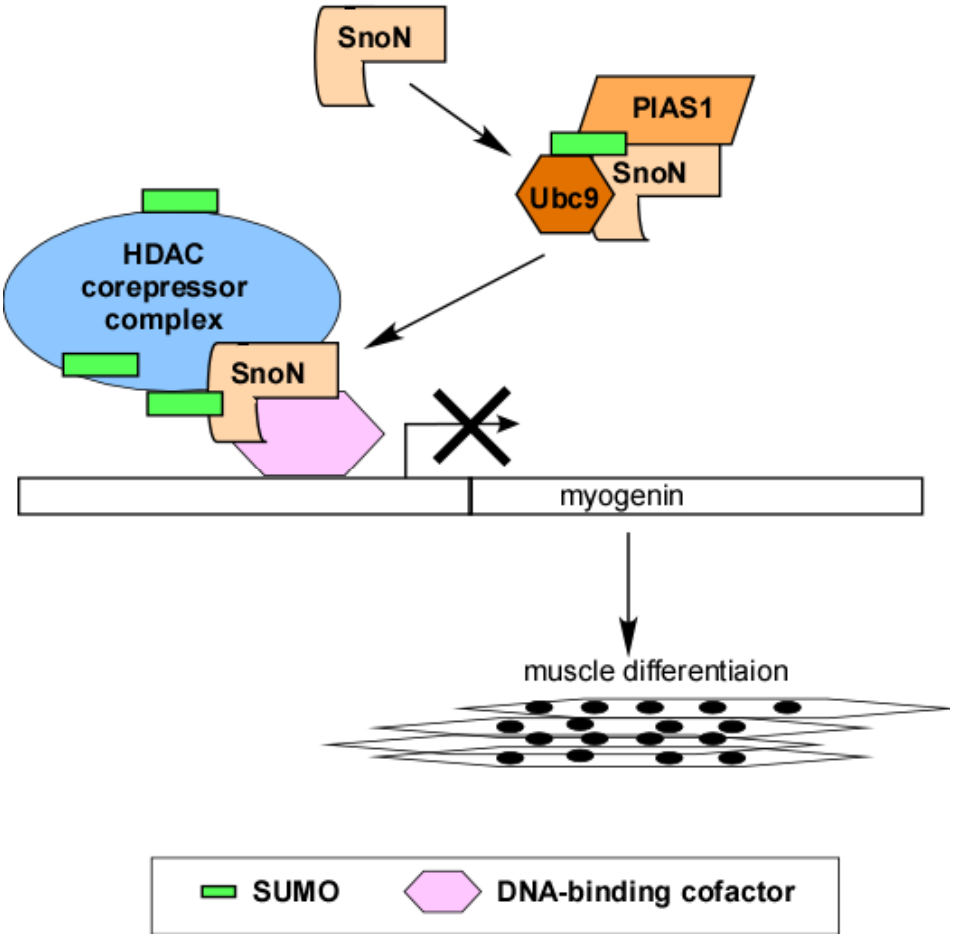


Figure 4-1 Model for the role of SnoN sumoylation in regulating Myogenin expression

Unsumoylated SnoN may not associate with the *myogenin*-promoter specific HDAC corepressor complex. SnoN sumoylation mediated by Ubc9 and PIAS1 may target the modified SnoN to vicinity of this *myogenin*-promoter specific HDAC corepressor complex. In doing this, SnoN may recruit this corepressor complex to the promoter of *myogenin*, resulting in transcriptional repression of Myogenin whose expression leads muscle differentiation.

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