Transcription factor Sp3 as target for SUMOylation *in vivo*

Dissertation

zur

Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

dem Fachbereich Biologie

der Philipps-Universität, Marburg/Lahn

vorgelegt von

Grigore Rişchitor

aus

Negreşti / Rumänien

Marburg, Februar 2005

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am:

Prufungvorsitzender Prof. Dr. Renate Renkawitz-Pohl
Betreuer der Arbeit Prof. Dr. G. Suske

Tag der mündlichen Prüfung:

Die Untersuchungen zur vorliegenden Arbeit wurden von August 2001 bis Dezember 2004 im IMT (Institut für Molekularbiologie und Tumorforschung) in Marburg unter der Leitung von Herrn Prof. Dr.Guntram Suske durchgeführt.

Ich versichere, dass ich meine Dissertation mit dem Titel “Transcription factor Sp3 as target for SUMOylation *in vivo*“ selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, 25 Februar 2005 Grigore Rischitor

Im Zusammenhang mit der Thematik der vorliegenden Dissertation wurden folgende Publikationen erstellt:

Sapetschnig A., Rischitor G., Braun H., Doll A., Schergaut M., Melchior F., Suske G. (2002). Transcription factor Sp3 is silenced through SUMO modification by PIAS1. EMBO Journal 21, 5206-5215.

Sapetschnig A., Koch F., Rischitor G., Mennenga T., Suske G. (2004). Complexity of translationally controlled transcription factor Sp3 isoform expression. The Journal of Biological Chemistry *279*, 42095-42105.

.

Contents

Abbreviations

All units of measurement are abbreviated according to the International System of units (SI).

A	Adenosine
Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumine
bp	basepair
C	Cytosine
CalBP	Calmodulin Binding Peptide
DAPI	4',6-Diamidino-2-phenylindole
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethylsulfoxid
DTT	1,4 Dithiothreitol
E1	SUMO or ubiquitin activating enzyme
E2	SUMO or ubiquitin conjugating enzyme
E3	SUMO or ubiquitin ligases
EDTA	Ethylenediamine tetraacetic acid
et al.	and others
EtOH	Ethanol
FCS	Fetal Calf Serum
Fig	Figure
G	Guanosine
GFP	Green Fluorescent Protein
EGFP	Enhanced Green Fluorescent Protein

HA	Hemagglutinin epitope
HRP	Horseradish peroxidase
K	Lysin
Kan	Kanamycin
kb	kilobases
kDa	Kilodalton
LB	Luria-Bertani-Medium
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PIAS	Protein Inhibitor of Activated STAT
PIC	Protease Inhibitor Cocktail
PML	Promyelocytic Leukemia protein
PMSF	Phenylmethylsulfonyl fluoride
rpm	rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
Sp1	Specificity protein1
Sp3	Specificity protein3
SUMO	Small Ubiquitin-like Modifier
T	Thiamine
TAP	Tandem Affinity Purification
TE	Tris-EDTA
TEMED	N, N, N‘, N‘-Tetramethylene diamine
TEV	Tobacco Etch Virus
U	Unit
Ubc9	Ubiquitin conjugating enzyme 9
wt	Wild type
www	World Wide Web

Summary

A group of sequence-specific DNA-binding proteins related to the transcription factor Sp1 (specificity protein 1) has been implicated in the regulation of many different genes, since binding sites for these transcription factors (GC/GT boxes) are a recurrent motif in regulatory sequences of these genes. In contrast to the transcriptional activators Sp1 and Sp4, the ubiquitously expressed Sp3 protein can both activate and repress transcription. The complex activity of Sp3 depends on two glutamine-rich activation domains, similar to those found in Sp1 and Sp4, and, adjacent to these, on an inhibitory domain unique to Sp3. The critical lysine residue in the Sp3 inhibitory domain lies within a consensus motif (IK551EE) that targets proteins for SUMO modification. SUMO (small ubiquitin-related modifier) is covalently attached to lysine residues in target proteins via an isopeptide linkage in a multi-step process that is analogous to ubiquitination. SUMOylation is a dynamic, reversible process, and distinct enzymes are responsible for adding or removing SUMO from target proteins.

The present work analyses various aspects of SUMO conjugation to Sp3 *in vivo*. Studying modification of Sp3 by SUMO is complicated by the existence of a number of Sp3 isoforms. Immunoblot analyses revealed four distinct Sp3 proteins, two slow migrating of more than 100 kDa and two fast migrating species. Seven to eight Sp3 bands appeared, when cells were lysed in denaturing conditions. The additional protein species represent SUMO modified Sp3 isoforms. Currently, it is not known whether the relative distribution of the different Sp3 isoforms is regulated. However, a significant shift towards the long isoforms of Sp3, however, is observed in Sp1-/- ES cells demonstrating that Sp3 isoform expression principally can change *in vivo*. In addition, this observation suggests that the long isoforms of Sp3 may take over Sp1 functions under Sp1 knockout conditions.

When Sp3 is overexpressed along with SUMO1 and SUMO2 in cells in culture, attachment of both SUMO paralogues to Sp3 occurred with almost equal efficiency.

Beside lysine 551 within the inhibitory domain, there are two other potential

SUMOylation sites in Sp3 (VKQE at position 9 and IKDE at position 120). This study revealed that SUMOylation takes place exclusively at K551, present in all four isoforms.

The Sp3 transcription factor is located predominantly in the nucleus. Visualization of endogenous Sp3 by immumofluorescence showed a sponge-like, diffuse appearance. Evolutionally closely related Sp family members Sp1 and Sp2 are also located in the nucleus and the subcellular localization patterns are similar to Sp3. Ectopic expression of SUMO1 fused to GFP (green fluorescent protein) led to the accumulation of this fusion protein within subnuclear "dots" or PODs (promyelocytic leukemia oncogenic domains), whereas endogenous Sp3 remained diffusely distributed throughout nuclei. In addition, the wild-type Sp3 isoforms and the SUMOylation-deficient mutants of Sp3were located in the nucleus exhibiting also a sponge-like, diffuse appearance.

Analyzing the Sp3 expression in different cell lines and mouse organs revealed that the relative level of Sp3 modification by SUMO is not cell line or organ dependent. In addition, no variation in Sp3 expression pattern after serum starvation, serum induction and heat shock was observed.

Ultraviolet radiation or Tumor Necrosis Factor alpha and Cycloheximide treatment of mammalian cells did not alter the SUMOylation level of Sp3 protein in our experimental conditions. A significant reduction in Sp3 SUMO modification was observed upon treatment with MG-132, a cell-permeable inhibitor of the proteasome. Possibly this proteasome inhibitor prevents proteasome degradation of SUMO specific isopeptidase, which subsequently remove the Sp3-SUMO moiety.

PIAS1 (protein inhibitor of activated STAT) was previously cloned in a two-hybrid screen by using the inhibitory domain of Sp3. Moreover, it was shown that PIAS1 strongly enhances SUMO-modification of Sp3 *in vitro* and thus acts as an SUMO E3 ligase towards Sp3. Nuclear extract fractionation studies suggested that PIAS1 is part of (a) high molecular weight complex(es). PIAS1-associated proteins might confer substrate specificity towards Sp3 and other transcription factors and/or regulate PIAS1 activity *in vivo*. For the purification and identification of PIAS1-associated proteins, a number of C-terminal tagged expression plasmids were constructed for constitutive

and inducible expression. The dual-tag affinity purification system established in this thesis work contains a small 15 amino acid artificial tag (BiotinTAG) that becomes biotinylated by the BirA ligase upon co-transfection of an appropriate expression construct. To enhance specificity, a second tag was included in the expression vectors (Calmodulin Binding Peptide or alternatively FLAG or Triple-FLAG). In addition, dual tags expression plasmids for Sp3 were constructed.

The establishment of stable cell lines expressing these fusion proteins in an inducible manner was initiated. Such cell lines might be ideal for further analyzes of PIAS1 activities and to purify PIAS1 (Sp3) associated factors.

Zusammenfassung

Bei einer Gruppe von sequenzspezifischen DNA-Bindeproteinen, die mit dem Transkriptionsfaktor Sp1 (Specificity Protein 1) verwandt sind, wird eine Beteiligung an der Regulation verschiedener Gene vermutet, da die Bindestellen dieser Transkriptionsfaktoren (GC/GC-Boxen) ein wiederkehrendes Motiv in regulatorischen Sequenzen von Genen darstellen. Im Gegensatz zu den transkriptionellen Aktivatoren Sp1 und Sp4 kann das ubiquitär exprimierte Sp3 die Transkription sowohl aktivieren, als auch reprimieren.

Die komplexe Aktivität von Sp3 ist abhängig von zwei glutamin-reichen Aktivierungs-Domänen, ähnlich denen von Sp1 und Sp4, sowie von einer neben den Aktivierungs-Domänen liegenden inhibitorischen Domäne, die nur in Sp3 gefunden wurde. Der kritische Lysin-Rest in der inhibitorischen Domäne von Sp3 liegt innerhalb einer Konsensus-Sequenz, welche das Ziel für die SUMOylierung des Proteins darstellt. SUMO (small ubiquitin-related modifier) wird in einem Mehrschrittprozeß analog der Ubiquitinierung über eine Isopeptid-Bindung mit einem Lysin-Rest des Zielproteins kovalent verknüpft. Die SUMOylierung ist ein dynamischer und reversibler Prozeß. Mehrere Enzyme sind für die Verknüpfung bzw. Loslösung von SUMO an bzw. von Zielproteinen verantwortlich.

In der vorliegenden Arbeit werden verschiedene Aspekte der SUMO-Konjugation an Sp3 *in vivo* analysiert. Die Untersuchungen zur SUMOylierung von Sp3 wurden durch die Existenz verschiedener Sp3 Isoformen verkompliziert. Die Immunoblot-Analyse deckte die Existenz vier verschiedener Isoformen auf: zwei im Gel langsam migrierende Formen mit einem Molekulargewicht über 100 kDa, sowie zwei schnell migrierende Formen. Es erschienen sieben bis acht Sp3-Banden, wenn die Zellen unter speziellen Bedingungen lysiert wurden. Die hier zusätzlichen Banden stellen die SUMOylierten Isoformen dar. Derzeit ist noch nicht bekannt, ob die relative Verteilung der verschiedenen Isoformen reguliert wird. Eine deutliche Verschiebung zu den langen Isoformen konnte in Sp1-/- ES-Zellen beobachtet werden, was darauf

hindeutet, daß die Expression der verschiedenen Sp3 Isoformen *in vivo* veränderbar ist. Außerdem läßt diese Beobachtung die Vermutung zu, daß die langen Isoformen von Sp3 die Funktionen von Sp1 unter Sp1-defizienten Bedingungen übernehmen.

Bei der Überexpression von Sp3 sowie SUMO1 und SUMO2 *in vivo* erfolgte die Bindung beider SUMO-Proteine an Sp3 mit ähnlicher Effizienz.

Neben dem Lysin-Rest an Stelle 551 innerhalb der inhibitorischen Domäne gibt es noch zwei weitere potentielle SUMOylierungsstellen in Sp3 (VKQE an Position 9 und IKDE an Position 120). Die vorligende Studie hat gezeigt, daß die SUMOylierung nur an K551 stattfindet, die in allen vier Isoformen vorhanden ist.

Der Transkriptionsfaktor Sp3 ist hauptsächlich im Nukleus lokalisiert. Die Visualisierung des endogenen Sp3 mit Hilfe der Immunfluoreszenz zeigte eine schwamm-ähnliche, diffuse Erscheinung. Die evolutionär eng verwandten Sp-Faktoren Sp1 und Sp2 sind ebenfalls im Nukleus lokalisiert und zeigen ähnliche subzelluläre Verteilungsmuster wie Sp3. Die Überexpression eines Fusionsproteins aus SUMO1 und GFP (green fluorescent protein) führte zur Akkumulierung dieses Fusionsproteins in subnuclearen PODs (promyelocytic leukemia oncogenic domains), während endogenes Sp3 weiterhin eine diffuse Verteilung im Nukleus zeigte. Die Wildtyp-Sp3 Isoformen und die Sp3 SUMOylierungs-defizienten Mutanten sind ebenfalls schwamm-ähnlich und diffus im Nukleus verteilt.

Die Analyse der Sp3-Expression in verschiedenen Zelllinien und Mausorganen zeigte, daß das Ausmaß der Sp3-Modifikation mit SUMO nicht zelllinien- bzw. organspezifisch ist. Außerdem führten Serum-Entzug, Serum-Induktion und Hitzeschock zu keinerlei Veränderungen im Sp3-Expressionsmuster.

Die ultraviolette Bestrahlung oder der Tumor Nekrose Faktor alpha und die Cycloheximid-Behandlung von Säugetierzellen-Zellen veränderten den SUMOylierungs-Gehalt von Sp3 unter den hier gestellten experimentellen Bedingungen nicht. Eine signifikante Reduktion der Sp3-SUMO-Modifikation zeigte sich nach der Behandlung mit MG-132, einem zell-permeablen Inhibitor des Proteasoms. Möglicherweise ist dieser Proteasom-Inhibitor mit dafür verantwortlich, daß SUMO-spezifische Proteasen nicht abgebaut werden und diese deshalb das

SUMOylierte Sp3 entfernen können.

PIAS1 (protein inhibitor of activated STAT) war in einem Yeast-Two-Hybrid Screen identifiziert, bei dem die inhibitorischen Domäne von Sp3 als Köder diente. Darüber hinaus wurde gezeigt, daß PIAS1 die SUMOylierung von Sp3 *in vitro* verstärkt und deshalb als SUMO-E3 Ligase bei der Sp3-SUMOylierung wirkt. Studien zur Fraktionierung von Kernextrakten deutenten darauf hin, daß PIAS1 *in vivo* Teil eines Komplexes mit hohem Molekulargewicht ist. PIAS1-assoziierte Proteine trugen möglicherweise zur Substratspezifität bei der Sp3-SUMOylierung bzw. SUMOylierung anderer Transkriptionsfaktoren bei und/oder regulierten zudem die Aktivität von PIAS1 *in vivo*.

Zur Reinigung und Identifizierung von PIAS1-assoziierten Proteinen wurde eine Anzahl C-terminal getagter Expressionsplasmide mit konstitutiver und induzierbarer Expression konstruiert. Das Dual-Tag Affinitäts-Reinigungs-System, das während dieser Arbeit etabliert wurde, beinhaltet ein kleines 15 Aminosäuren-umfassendes artifizielles Tag (BiotinTAG), welches von der BirA Ligase nach Kotransfektion eines geeigneten Expressionskonstruktes biotinyliert werden kann. Um die Spezifität zu erhöhen, wurde ein zweites Tag in die Expressionsvektoren kloniert (Calmodulin Bindepeptid oder alternativ FLAG oder Triple-FLAG). Zusätzlich erfolgte die Konstruktion von Dual-Tag-Expressionsplasmiden für Sp3. Die Etablierung von stabilen Zelllinien, die diese Fusionsproteine induzierbar exprimieren, wurde initiiert. Derartige Zelllinien wären ideal für weitere Analysen der Aktivität von PIAS1 und für die Reinigung von PIAS1 (Sp3) assoziierten Faktoren.

1. Introduction

1.1. Properties of Sequence-Specific DNA Binding Transcription Factors

In eukaryotes, there are tens of thousands of protein-coding genes, each of which has its own specific program of transcriptional control. Much of the specificity of these programs is regulated by sequence-specific DNA binding proteins that bind to the proximal promoter and distal transcriptional regulatory regions (such as enhancers and silencers). Sequence-specific DNA binding transcription factors (henceforth termed "sequence-specific factors") interpret and transmit the information that is encoded in the primary DNA sequence to the factors and cofactors that mediate the synthesis of RNA transcripts from the DNA template (Kadonaga, 2004). Thus, the sequence-specific factors collectively function as the key interface between genetic regulatory information and the transcription system (Fig.1.1).

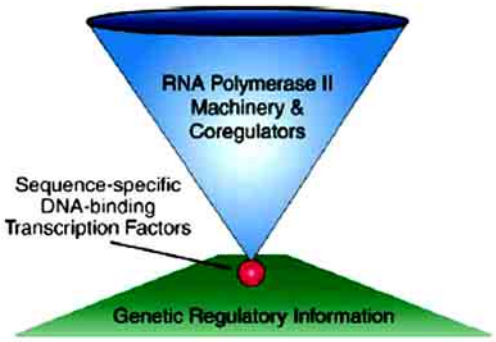


Fig.1.1. Sequence-Specific DNA Binding Transcription Factors Interpret and Transmit Genetic Regulatory Information.
In this diagram, sequence-specific factors are depicted as the apex at the interface of the vast array of genetic regulatory information and the inverted cone of the RNA polymerase II transcriptional machinery and coregulators.
(After Kadonaga, 2004).

Transcription is succinctly defined as the process of RNA synthesis complementary to a DNA template. Transcription is a complex process that relies on the collective action of sequence-specific factors along with the core RNA polymerase II

transcriptional machinery, an assortment of coregulators that bridge the DNA binding factors to the transcriptional machinery, a number of chromatin-remodeling factors that mobilize nucleosomes, and a variety of enzymes that catalyze the covalent modification (e.g., acetylation, deacetylation, phosphorylation, dephosphorylation, methylation, ubiquitination, deubiquitination, SUMOylation and ADP-ribosylation) of histones and other proteins.

1.1.1. Sequence-Specific Transcription Factors Are Modular

A typical sequence-specific factor has a DNA binding module linked to one or more activation or repression modules as well as in certain cases, a multimerization module and a regulatory module (Fig.1.2.-A).

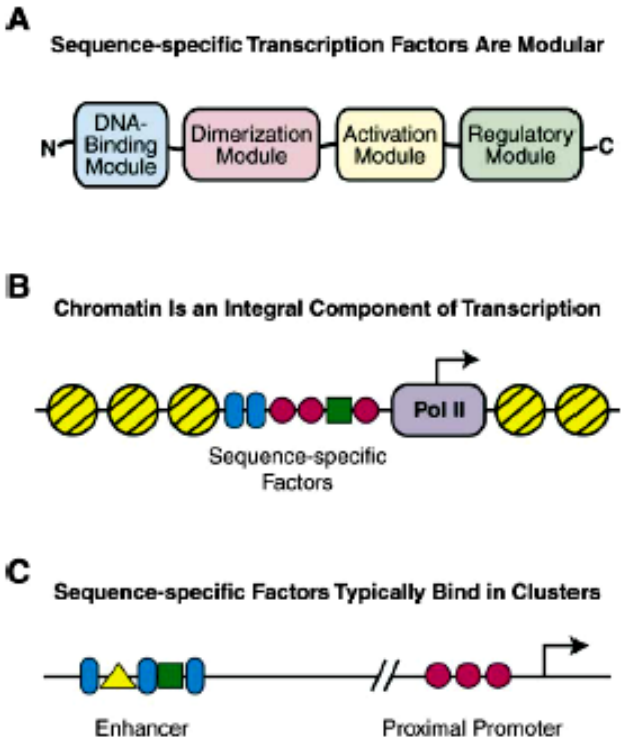


Fig.1.2. Some properties of sequence-specific DNA Binding transcription factors
A. Sequence-specific factors are composed of functional modules.
B. Chromatin is an integral component in the function of sequence-specific factors. Nucleosomes are depicted in yellow.
C. Recognition sites for sequence-specific factors tend to be located in clusters
(After Kadonaga, 2004).

The first well-defined DNA binding module was the helix-turn-helix motif, which was originally discovered in prokaryotic DNA binding proteins. It later became apparent, however, that nature has generated many distinct DNA binding modules, which include the homeodomains (a variant of the helix-turn-helix), zinc fingers (of which there are different types), leucine zippers, helix-loop-helix motifs, HMG1 domains and others (Pabo and Sauer, 1992). Both the leucine zipper (Landschultz et al., 1988) and helix-loop-helix (Murre et al., 1989) motifs with their associated basic regions are able to perform dual DNA binding and dimerization functions. The first of the nonacidic activation regions was found in transcription factor Sp1 (Specificity protein1), which contains multiple glutamine-rich activation motifs (Courey and Tjian, 1988). Other transcriptional activation motifs include proline-rich regions (Mermod et al., 1989) and hydrophobic β sheets (Leuther et al., 1993; Van Hoy et al., 1993).

Some transcription factors are controlled by regulatory modules. For example, nuclear receptors are transcription factors that contain a regulatory module that is located on the same polypeptide as the DNA binding and transcriptional activation modules. IkB is an example of a regulatory module that is not covalently attached to the transcription factor (NF-kB proteins) that it regulates. Instead, IkB functions as a detachable regulatory subunit that modulates the activity and cellular location of NF-kB (Baeuerle and Baltimore, 1988).

1.1.2. Sequence-Specific Factors Regulate Transcription via Recruitment of Coactivators and Corepressors

How do sequence-specific factors work? Current evidence indicates that the sequence-specific factors function mainly by recruitment of transcriptional coactivators and corepressors to the DNA template via protein-protein interactions (Ptashne and Gann, 1997). These cofactors then act both directly and indirectly to regulate the activity of the RNA polymerase II transcriptional machinery at the core promoter.

Many but not all of these coactivators and corepressors are recruited to the DNA template via interactions with the sequence-specific factors. Some coregulators are direct intermediaries between the sequence-specific factors and the general/basal

transcriptional machinery. For instance, there are specific interactions between sequence-specific factors and TAF's (TBP-associated factors) subunits of the TFIID component of the basal machinery. In addition to TAFs, many other coactivator complexes (which include TRAP, SMCC, Mediator, SRB complex, CRSP, DRIP, NAT, p300/CBP, and others) can serve as a bridge between the sequence-specific factors and the general/basal transcriptional machinery.

Another distinct class of cofactors are chromatin-related coregulators, which are also thought to be recruited by the sequence-specific factors. The chromatin-related coregulators affect transcription indirectly by remodeling nucleosomes or by covalent modification of histones (e.g., by acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation) or the DNA template (McKenna and O'Malley, 2002; Narlikar et al., 2002; Freiman and Tjian, 2003).

Other coregulators are not recruited by the sequence specific factors, but instead interact directly with RNA polymerase II and modulate the efficiency of transcriptional elongation (Hampsey and Reinberg, 2003).

1.1.3. Sequence-Specific Factors Can Be Regulated by Posttranslational Modifications

Some sequence-specific factors have been found to be regulated by posttranslational modifications. An excellent example is the phosphorylation of CREB (cyclic AMP response element binding protein) (Gonzalez and Montminy, 1989). CREB activates transcription of cyclic AMP-inducible genes via binding to CRE (cyclic AMP response element) motifs. First, cAMP stimulates protein kinase A, which phosphorylates CREB at serine residue 133. Upon phosphorylation, CREB becomes activated and stimulates transcription from cAMP-responsive promoters. Another example of posttranslational modification of transcription factors is the O-linkage of N-acetylglucosamine in many sequence-specific factors, which include Sp1, AP-1, AP-2, CTF/NF-I, Zeste, GAGA factor, and Adf-1 (Jackson and Tjian., 1988). The function of the O-linked N-acetylglucosamine monosaccharide residues remains to be clarified. Some results suggest a role of this modification in transcriptional repression

(Yang et al., 2002). Sequence-specific factors can also be acetylated. For example, acetylation of p53 increases the affinity of its binding to DNA (Gu and Roeder, 1997). Many transcription factors, which include the sequence-specific proteins, are ubiquitinated (Freiman and Tjian, 2003) or are modified by ubiquitin family proteins (Hilgarth et al., 2004; Watts, 2004).

1.1.4. Sequence-Specific Factors Are Members of Multiprotein Families

Many sequence-specific factors are members of multiprotein families. For instance, nuclear receptors are members of a superfamily of related proteins (Evans, 1988). AP-1 consists of Fos, Jun, and many other Fos and Jun related proteins as well as ATF and CREB-like proteins. CTF/NF-I is a family of proteins that appear to result from alternative RNA splicing (Santoro et al., 1988). NF-kB is yet another family of proteins (Baldwin, 1996). p53, p63, and p73 are also a protein family (Yang et al., 2002). Even the original Sp1 has turned out to be a member of the Sp family of proteins (Suske et al., 2005). Thus, there are many families of transcription factors. Within each family, the members often display closely related or essentially identical DNA binding properties but distinct activation functions. It remains a significant challenge to elucidate the molecular bases for the unique functional specificities of individual members of each transcription factor family.

1.1.5. Chromatin Is an Integral Component in the Function of Sequence-Specific Factors

Chromatin is the natural state of the DNA template. For many years, chromatin had been commonly viewed as an unimportant subject area with no role in general understanding of gene regulation. One early concept was that transcription factors function primarily to counteract chromatin-mediated global repression of basal transcription in the absence of activators. This “antirepression” model is distinct but not mutually exclusive from a “true activation” model in which sequence-specific factors function to increase the rate of the intrinsic transcription process (Adams and Workman, 1993; Paranjape et al., 1994).

Current data support both of these hypotheses, but also reveal an unforeseen complexity in the involvement of chromatin in transcriptional regulation that includes chromatin-remodeling factors (ATP-dependent enzymes that mobilize nucleosomes) and a variety of histone-modifying enzymes. It appears that sequence-specific factors recruit chromatin-remodeling factors and histone-modifying enzymes, which in turn function to rearrange chromatin structure (for instance, to relieve chromatin-mediated repression) as well as to modify histones in a specific fashion that promotes the desired gene activation or repression (Li, 2002).

1.1.6. Recognition Sites for Sequence-Specific Factors Tend to Be Located in Clusters

Individual eukaryotic sequence-specific factors generally bind to DNA with relatively low specificity. Thus, the precise control of gene transcription requires a higher degree of specificity than that typically afforded by the binding of a single sequence-specific factor to DNA. Instead, the high degree of specificity and potency of promoter and enhancer binding factors appears to be accomplished by the utilization of multiple factor recognition sites in composite cis-regulatory arrays (Fig.1.2. C). Hence, a cluster of several short (about 6 to 8 bp) recognition sites, such as in an enhancer region, would be rarely encountered in the genome, even though a single recognition site might be common. It is also relevant to note that multiple sequence-specific factors in a cluster typically function synergistically and activate transcription more strongly than each factor on its own (Laybourn and Kadonaga, 1992).

In this manner, the specificity of gene activation by sequence-specific factors derives from the use of multiple, clustered cis binding sites in conjunction with the synergistic enhancement of transcription that is achieved with multiple trans-acting factors.

1.1.7. Other Properties of Sequence-Specific Transcription Factors

First, sequence-specific factors have been found to interact with transcriptional insulator (also known as “boundary”) elements, which function to block the spreading

of the influence of either positive DNA elements (such as enhancers) or negative DNA elements (such as a silencers, or heterochromatin-like repressive effects) (Bell et al., 2001). Sequence-specific factors that have been found to act at insulator elements include BEAF-32, suppressor of Hairy-wing and Zw5 in *D. melanogaster* and CTCF in mammalians (Moon at al., 2004). It will be important to determine the specific features of these factors that enable them to function in transcriptional insulation.

Second, sequence-specific activators can stimulate transcription initiation as well as elongation (Blau et al., 1996). In fact, some factors primarily stimulate initiation, whereas other factors stimulate predominantly elongation or both initiation and elongation.

Third, a subset of sequence-specific activators, which include Sp1 and CBF/NF-Y, are commonly found in the proximal promoter region of genes (-250 to -30 relative to the +1 transcription start site). Some of these proximal promoter factors might function most effectively near the start site because they interact with the core transcriptional machinery. Moreover, some of the promoter proximal factors might also act as a conduit between distal enhancers and the basal/general transcriptional machinery, possibly as tethering factors that link or loop enhancer complexes to the core transcription complex (Calhoun et al., 2002).

1.2. Basic Classification of Sp Family Transcription Factors

1.2.1. The Sp/KLF Zinc Finger Transcription Factors

The zinc finger transcription factors are the most widely evolved family of transcription factors in eukaryotes. Among these factors, the Sp/KLF (Specificity Protein/Krüppel-Like Factor) family of transcription factors has received attention due to important roles in development, differentiation, and oncogenic processes (Suske, 1999; Philipsen and Suske, 1999; Bouwman and Philipsen, 2002).

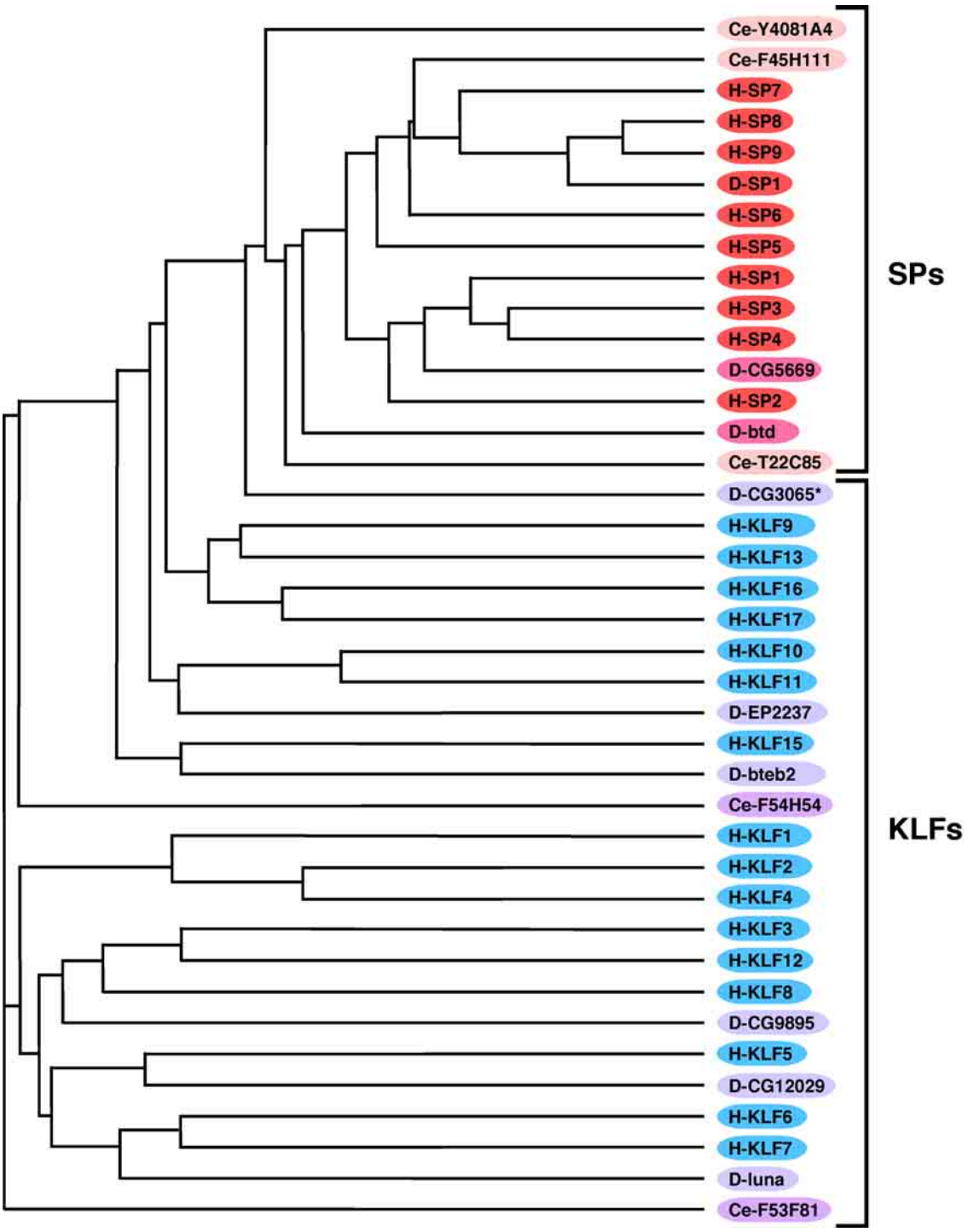


Fig.1.3 Relationships between the SP and KLF factors of human, *Drosophila* and *C. elegans*

Of each factor, the 110 aa domain containing the BTD/zinc finger motifs was used for the multiple alignment with ClustalW (http://www.ebi.ac.uk/clustalw/). This alignment was used to construct the cladogram.

*: The current sequence of *C. elegans* F45H11.1 only contains the BTD motif and the first finger.

**: one amino acid was removed from finger 3 of Ce-T22C8.5 (HXXXH instead of

HXXXXH), because the extra amino acid is not handled appropriately in the multiple alignment generated with ClustalW.
***: The sequence of D-CG3065 is deduced from the *Drosophila* genome sequence; the current annotation does not contain the complete BTD/zinc finger motif.
(After Suske et al., 2005).

For long it has been known that the general transcription factor Sp1 (Specificity protein 1) can bind and act through GC/GT boxes, and Sp1 was therefore thought to be an extremely versatile protein essential for many different functions of the mammalian nucleus. More recently, however, it has become clear that Sp1 is not the only transcription factor binding and acting through these elements. It simply represents the first identified and cloned protein of a new and still growing family of transcription factors. Family members contain a highly conserved DNA-binding domain consisting of three zinc fingers. The availability of near complete genome sequences of mouse and human greatly facilitates the unambiguous assignment of names to all the members of the SP/KLF family (Suske et al., 2005). Currently this family of transcription factors comprises at least 25 different mammalian members (Fig.1.3).

The 81 amino acid DNA-binding domain that is found close to the C-termini of all members essentially defines the Sp/XKLF family of transcription factors. It consists of three C2H2-type zinc fingers arranged similar to those found in the *Drosophila melanogaster* regulator protein Krüppel. Accordingly, some of the proteins have been named Krüppel-like factors.

The striking similarity of the linker amino acids between the individual fingers as well as the identical length of the DNA-binding domain strongly suggest that the higher order structure of the three fingers is crucial for the biological function of the proteins (Fig.1.4). Structural studies on zinc finger peptides bound to DNA have provided information that allows predictions on the DNA sequence recognized by finger domains. The amino acids of the Sp1 zinc fingers that are most likely to make specific contacts with the DNA are the amino acids KHA within the first, RER within the second and RHK within the third zinc finger domain. These critical amino acids are conserved in Sp3, Sp4, BTEB1, TIEG1 and TIEG2 proteins (Fig.1.4). Consistent with

this conservation, Sp3, Sp4, BTEB1 and TIEG2 recognize classical Sp1-binding sites. In addition, the relative affinity for the GC box is very similar, if not identical, between these proteins. GT or CACCC boxes are also recognized by these proteins but with slightly lower affinities. In Sp2, a leucine residue replaces the critical histidine residue within the first zinc finger (Fig.1.4).

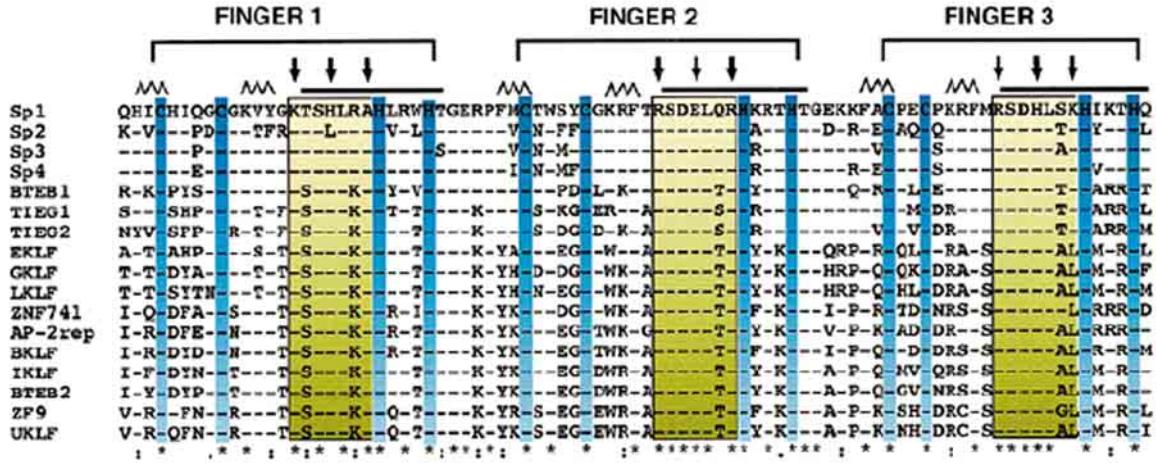


Fig.1.4. Protein sequence alignment of the zinc finger domains of mammalian Sp/XKLF family members.
All sequences are human sequences with the exception of LKLF, AP-2rep, BKLF and IKLF, which are of mouse origin. The cysteine and histidine residues that are involved in zinc coordination are in blue. Arrows point to the amino acid positions that probably determine the recognition specificity of the fingers by contacting specific bases of the DNA. Black lines indicate a-helices; b-sheets are shown as zig-zag lines. The amino acids that are thought to make base contacts are boxed. Residues conserved between all family members are indicated (*).
(After Philipsen and Suske, 1999).

Contrary to initial expectations that this family of factors would likely have redundant functions, they in fact have important individual biological functions as shown by gene knockout studies (e.g. EKLF/KLF1, LKLF/KLF2 and KLF5). However, the underlying mechanisms governing their specific functions and regulation are poorly understood.

Within the Sp factors, Sp1, Sp2, Sp3, and Sp4 form a subgroup based on their similar modular structure (Fig.1.5). Sp1, Sp3, and Sp4 contain two major

glutamine-rich transactivation domains A and B that are essential for transcriptional activation. Next to these A and B domains, serine/threonine-rich sequences are located that may be a target for post-translational modification.

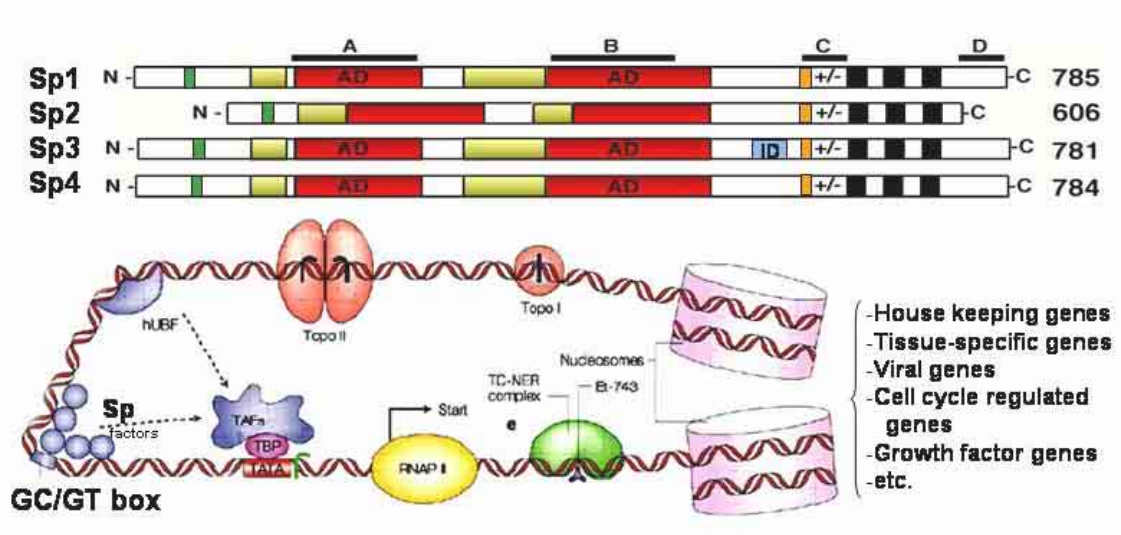


Fig.1.5. Structural motifs in Sp factors. Sp-proteins bind to GC/GT boxes and are involved in the expression of many genes including house keeping genes, tissue-specifically expressed genes, viral genes, cell-cycle-regulated genes as well as growth factor genes. The upper drawing depicts schematically the four human Sp-family members Sp1, Sp2, Sp3 and Sp4. Their length in amino acids is indicated on the right. The length of Sp3 refers to the full-length isoform. Red boxes indicate activation domains that are rich in glutamine residues and yellow boxes indicate serine/threonine-rich regions. Sp and Btd boxes are marked by green and orange boxes, respectively. The region preceding the first zinc finger (+/-) is rich in charged amino acids. The black boxes represent the zinc fingers. ID in the blue box indicates the inhibitory domain of Sp3 that contains the IKEE SUMOylation site.
Abbreviations: topoisomerase (topo)-II–DNA or topo-I–DNA complexes; human upstream binding factor (hUBF); transcription-coupled nucleotide excision repair (TC-NER); TATA-binding protein (TBP)–TATA box complex; transcriptional activation factors (TAFs); RNA polymerase II (RNAP II).

All the Sp transcription factors contain a so-called Buttonhead box immediately N-terminal to the zinc finger domain This conserved stretch of 11 amino acid residues was originally identified in the *Drosophila* Sp1 homologue Buttonhead (Btd) (Wimmer et al., 1996). It may contribute to the transactivation potential of the factors, since a deletion of an overlapping region results in reduced activity of Sp1 in vitro (Courey and Tjian, 1988). Furthermore, domain C (Yieh et al., 1995), and more specifically the

Btd element within domain C (Athanikar et al., 1997), are involved in synergistic activation by Sp1 or Sp3 with sterol-regulatory element-binding proteins (SREBP). Harrison and coworkers (2000) identified another stretch of conserved amino acids consisting of the sequence SPLALLAATCSR/KI (Sp box) that is located at the N-terminus of the proteins. This element contains an endoproteolytic cleavage site and is situated close to a region at the N-terminus of Sp1 that targets proteasome-dependent degradation in vitro (Su et al., 1999). Although not required to direct cleavage, the fact that the Sp box is highly conserved indicates that it may have a function in regulation of proteolysis of Sp factors. Another possible role for the Sp box may lie in the control of transactivation potential via interaction with a putative repressor (Murata et al., 1994). Although the functions of the Btd and Sp boxes are not clear at the moment, their absence in the XKLF subgroup confirms the relationship between the Sp transcription factors.

1.2.2. Sp3: Activator versus Repressor

Unraveling the transcriptional role of Sp3 was complicated by the fact that four Sp3 isoforms exist, two 110-115 kDa Sp3 proteins and two approximately 60-70 kDa Sp3 species observed in immunoblots. Four isoforms of Sp3 are expressed *in vivo* that differ in the extent of the amino terminal part. Detailed mutational analyses suggest that all four isoforms derive from alternative translational start sites. Moreover, an upstream open reading frame regulates expression of the two long isoforms (Sapetschnig et al., 2004).

Reports on the transcriptional properties of Sp3 appear, at first sight, contradictory. Sp3 has been shown to act as a transcriptional activator similar to Sp1 (Udvadia et al., 1995; Ihn and Trojanowska, 1997; Ding et al., 1999). In other experiments, Sp3 remained inactive or acted only as a very weak activator (Hagen et al., 1994; Majello et al., 1994; Dennig et al., 1995 and others). Most of these reports are based on co-transfection experiments into the insect cell line SL2. Usually, a distinct promoter fragment containing appropriate Sp-binding sites fused to a reporter gene was co-transfected along with Sp1 and Sp3 or both together. If Sp3 is expressed to the same

extent as Sp1 but does not act as a strong activator, it will compete for the same binding site and thus lower Sp1-mediated activation. The experimental conditions which are needed for Sp3 to act as a strong activator or a transcriptional inactive molecule which represses Sp1-mediated activation are not completely understood. The structure and the arrangement of the recognition sites appear to determine whether Sp3 is transcriptionally inactive and can repress Sp1-mediated activation or whether it acts as a strong activator. Promoters containing a single binding site are activated, whereas promoters containing multiple binding sites often are not activated or respond weakly to Sp3 (Birnbaum et al., 1995; Dennig et al., 1996). Purified recombinant Sp3 expressed in SL2 cells (Braun and Suske, 1999) act in an *in vitro* system as strong activator similar to Sp1 (Braun et al., 2001). Whether Sp3 acts as an activator or as a repressor of Sp1-mediated activation might also depend of the cellular context. Transfected Sp3 stimulated transcription from the HERV-H long-terminal repeat in the teratocarcinoma cell line NTera2-D1 but acted as a repressor in HeLa and insect cells (Sjottem et al., 1996).

It has been suggested that the two small Sp3 isoforms might act as repressor molecules whereas the full length Sp3 isoform does act as an activator (Kennett et al., 1997). Although attractive and simple, this model does not seem to hold true. Exclusive expression of full length Sp3 triggered by an artificial leader sequence can also represses Sp1-mediated activation (Dennig et al., 1996). It is clear that both N-terminal glutamine-rich regions can act as strong activation domains on their own in both insect and in mammalian cells (Dennig et al., 1996; Majello et al., 1997).

The molecular basis for the inactivity of Sp3 under certain conditions has been mapped to an inhibitory domain located between the second glutamine-rich activation domain and the first zinc finger. The amino acid triplet KEE within this domain is absolutely essential for repressor function (Dennig et al., 1996). Mutation of these amino acids to alanine residues converted almost inactive Sp3 to a strong activator. The inhibitory domain of Sp3 acts as an independent module in *cis*. It can be transferred to other activation domains which in turn lose their activation properties (Dennig et al., 1996).

This domain resides in a highly charged stretch of amino acids that is not present in the comparable region of Sp1 (domain C). It resembles repressor domains present in C/EBPa, C/EBPb (Angerer et al., 1999), c-Fos and FosB (Brown et al., 1995). The presence of a repressive module explains the earlier observed inactivity of the N-terminal region of Sp3, despite the presence of glutamine-rich domains that resemble the Sp1 transactivation domains A and B (Hagen et al., 1994). Mutation of a critical KEE amino acid triplet results in relief of repression and potentates Sp3 transactivation, especially of promoters containing multiple binding sites (Dennig et al., 1996). Furthermore, it was shown that the lysine residue K551 within inhibitory domain is a target for modification by SUMO (Sapetschnig et al., 2002).

1.3. SUMO Small Ubiquitin-like MOdifier

A multitude of mechanisms determine the *in vivo* function of proteins. Among them are the regulation of protein levels via control of expression levels and turnover and regulation of protein activity, and localization and/or interactions by constitutive or reversible post-translational modifications. These modifications, usually accomplished via enzymatic reactions, result, for example, in acetylation, methylation, phosphorylation, ADP ribosylation, carboxylation, adenylylation, and glycosylation or prenylation of amino acid side chains. Among these is also ubiquitination, a post-translational modification that was first discovered in 1987 and has since been extensively studied (Bonifacino and Weissman, 1998; Hershko and Ciechanover, 1998).

Ubiquitination is the enzymatically catalyzed formation of an isopeptide bond between the C terminus of the 9-kDa polypeptide ubiquitin and ε-amino groups in lysines of the acceptor proteins. In principle, the modification is reversible, because the ubiquitin moiety can be removed from the acceptor molecule by deubiquitinating enzymes (isopeptidases). Ubiquitination is best known for its role in regulated protein degradation via the 26S proteasome. Commitment of a protein to the ubiquitin-dependent degradation pathway involves assembly of a polyubiquitin chain

on the target, usually via isopeptide bonds between lysine 48 of one ubiquitin and the C-terminal glycine residue of the neighboring ubiquitin. Although tagging a protein with ubiquitin chains usually leads to its complete degradation, it is sometimes also used for controlled activation of the protein via limited proteolysis. In addition, monoubiquitination plays a role in receptor mediated endocytosis (Hicke, 1997).

A number of proteins related to ubiquitin have been isolated over the years. These proteins fall into two groups, proteins that are not available for conjugation (e.g. Rad23, Dsk2p, Elongin B), and proteins that, like ubiquitin, are attached to other proteins (Ciechanover, 1998; Hodges et al., 1998; Hochstrasser, 2000; Jentsch and Pyrowolaski, 2000). To this second group belong the interferon-inducible ubiquitin cross-reacting proteins UCRP/ISG15, Nedd8, and SUMO1, which are 36, 57, and 18%, respectively, identical to ubiquitin at their primary sequence. Whether the 21-kDa yeast protein Apg12 that is required for autophagy in yeast, also belongs to this family or whether it is the first member of a new group of attachable proteins is presently unclear. Although Apg12 does not show any discernible homology to ubiquitin at the level of its primary sequence, it is attached to Apg5 by a mechanism that closely resembles ubiquitination (Ohsumi, 1999).

The significance of ubiquitin-related proteins remained rather obscure until the first targets, the SUMO1 target RanGAP1 (Matunis et al., 1996), and the Nedd8/Rub1 target Cdc53 (Lammer et al., 1998) were discovered. Only than did it becomes clear that these proteins are not simply variations on the ubiquitin theme, they play important role outside of protein degradation. Unlike the ubiquitin system, which primarily targets substrate proteins to the proteasome, SUMO1 conjugation has diverse cellular functions being implicated in a vast number of cellular processes, including nuclear transport, signal transduction, apoptosis, autophagy, cell cycle control, and regulation of ubiquitin-dependent degradation (Fig.1.6).

1.3.1. What is SUMO?

SUMO is the acronym for small ubiquitin-like modifier, also known as sentrin, GMP1, UBL1 and PIC1. SUMO1 family members are expressed throughout the

eukaryotic kingdom.

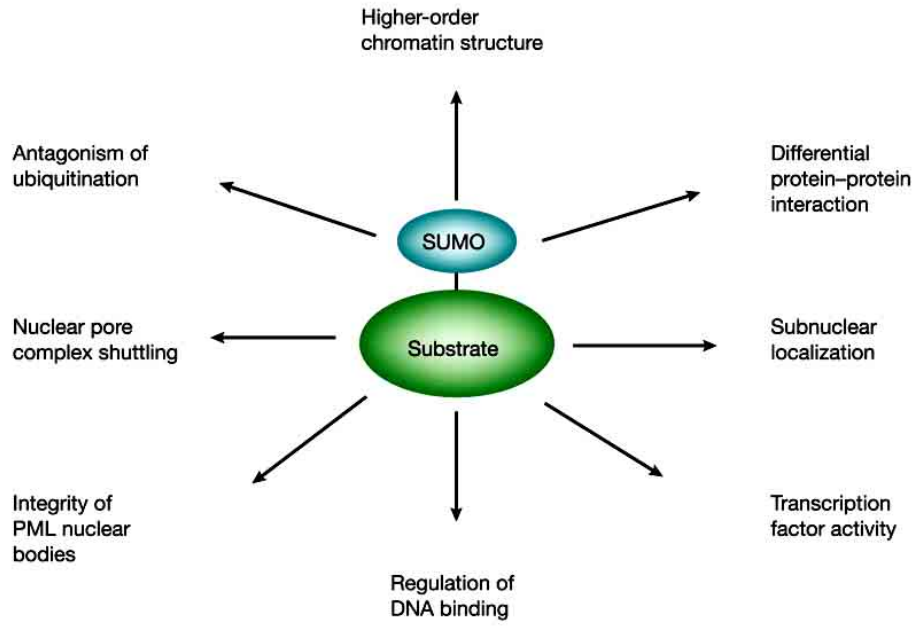


Fig.1.6. Signaling function of SUMO. Some of the known functions of SUMOylation are indicated with respect to transcriptional regulation.
(After Verger et al., 2002)

Members of the SUMO protein family appear to be present in protozoa, metazoa, plants, and fungi (Melchior, 2000). SUMO proteins from metazoa can be divided into two families: SUMO1 proteins and SUMO2/SUMO3 proteins. Within a given species, SUMO1 and SUMO2/3 proteins are about 50% identical to each other. Plant SUMO proteins and SUMO proteins from fungi and yeast fall into two groups distinct from both SUMO1 and SUMO2/3 proteins. *S. cerevisiae* contains a single essential SUMO gene, SMT3 (Johnson et al., 1997). In contrast, the *Schizosaccharomyces pombe* homolog Pmt3 is not truly essential (Tanaka et al., 1999). However, disruption leads to severe growth defects and phenotypes such as aberrant mitosis, increase in telomere length, and defects in chromosome segregation.

Analysis of an expressed sequence tag (EST) and genomic databases indicates the presence of at least one SUMO family member in *Aspergillus nidulans, Botrytis cinerea, Dictyostelium discoideum, Candida albicans and C. trypanosoma*. The

best-characterized plant SUMO protein is T-SUMO from tomato (Hanania et al., 1999). It was identified through its interaction with ethylene-inducing xylanase from the fungus *Trichoderma viride* and has been implicated through antisense experiments in plant defense responses that lead to programmed cell death. ESTc-DNAs encoding SUMO proteins have been found in libraries from many plant species (e.g. soja, maize, rice, pine tree, cedar, etc), and was reported the presence of at least three different expressed SUMO species in *Arabidopsis*. Zebrafish and *Xenopus laevis* have both SUMO1 and SUMO2/3 proteins. *Caenorhabditis elegans* has apparently only a SUMO1 family member (Choudhury and Li., 1997), whereas silk worm expresses cDNAs for a SUMO2/3 protein. A *Drosophila melanogaster* SUMO2 protein has been described (Bhaskar et al 2000, Huang et al 1998), but there is currently no strong evidence for a SUMO1 in this organism. While Northern blot analysis revealed two different SUMO transcripts in *Drosophila* embryos, larvae, and adult tissue, it is currently unknown whether these derive from the same SUMO genes. Both mice and humans have at least three different SUMO proteins, SUMO1, SUMO2, and SUMO3. Although SUMO2 and SUMO3 are very similar at the amino acid level (87% sequence identity for the human proteins), they are only 47% identical to SUMO1. Transcripts for SUMO1, SUMO2, and SUMO3 can be detected in all human and mouse tissues, indicating that these proteins are ubiquitously expressed (Chen et al., 1998; Howe et al., 1998).

SUMO4 was discovered recently as potential transcript with homologies to the SUMO gene family, after database searches in intron 6 of MAP3K7IP2 gene (Guo et al., 2004). SUMO4 has a restricted pattern of expression with highest levels reported in the kidney (Bohren et al., 2004). Currently, the mechanisms that determine selective modification by specific SUMO isoforms are not known, and the functional significance of modification by specific SUMO isoforms also remains to be determined.

Compared to posttranslational modifiers such as a phosphate or acetyl group, ubiquitin and ubiquitin-related proteins are structurally complex. Specific surface residues of ubiquitin participate in different ubiquitin dependent interactions such as

binding to the proteasome or components of the endocytic machinery (Sloper-Mould et al., 2001). NMR (nuclear magnetic resonance) studies have shown that SUMO1 has a similar protein fold. Importantly, despite the similar protein fold, the distribution of charged residues on the surface of SUMO is very different from that of ubiquitin or other ubiquitin-like proteins. In addition, SUMO has an N-terminal extension not found in ubiquitin (Fig.1.7). These differences likely account for the finding that distinct enzymes mediate SUMO conjugation and deconjugation as well as the unique activities attributed to SUMO.

Alignments of ubiquitin and SUMO1 indicate that although only 18 % identical in amino acid sequence, these two proteins have remarkably similar secondary structures. In addition, 3D protein backbone overlays of their core structures show that the tertiary structures of ubiquitin and SUMO1 bear close resemblance to each other. However, there are significant differences between the two proteins. A flexible 20-residue N-terminal extension in SUMO1 is absent from ubiquitin and the two proteins also have a dramatically different surface charge distribution (Fig.1.7).

SUMO is conjugated to target proteins by a pathway that is distinct from, but analogous to, ubiquitin conjugation (Fig.1.8). The same pathway appears to be used by SUMO1, -2 and -3 (Johnson and Gupta, 2001).

In SUMOylation, the target lysine generally falls within a recognizable consensus, namely φ -Lys-X-Glu (where φ is a large hydrophobic amino acid, most commonly isoleucine or valine, and X is any residue).

SUMO is first activated by a heterodimeric E1 enzyme (SAE1–SAE2 in humans or Uba2–Aos1 in *S. cerevisiae*) that uses ATP to adenylate the C-terminal glycine residue of SUMO. A thioester bond is then formed between the C-terminus of SUMO and a cysteine residue in SAE2, releasing AMP. In a transesterification reaction, SUMO is then transferred from SAE to the E2 SUMO-conjugating enzyme Ubc9.

In yeast, two SUMO proteases have been identified, Ulpl and Ulp2/Smt4, both of which are specific for SUMO and display compartmentalization, with Ulp1 being present at the nuclear pore complex and Upl2/Smt4 present in the nucleoplasm.

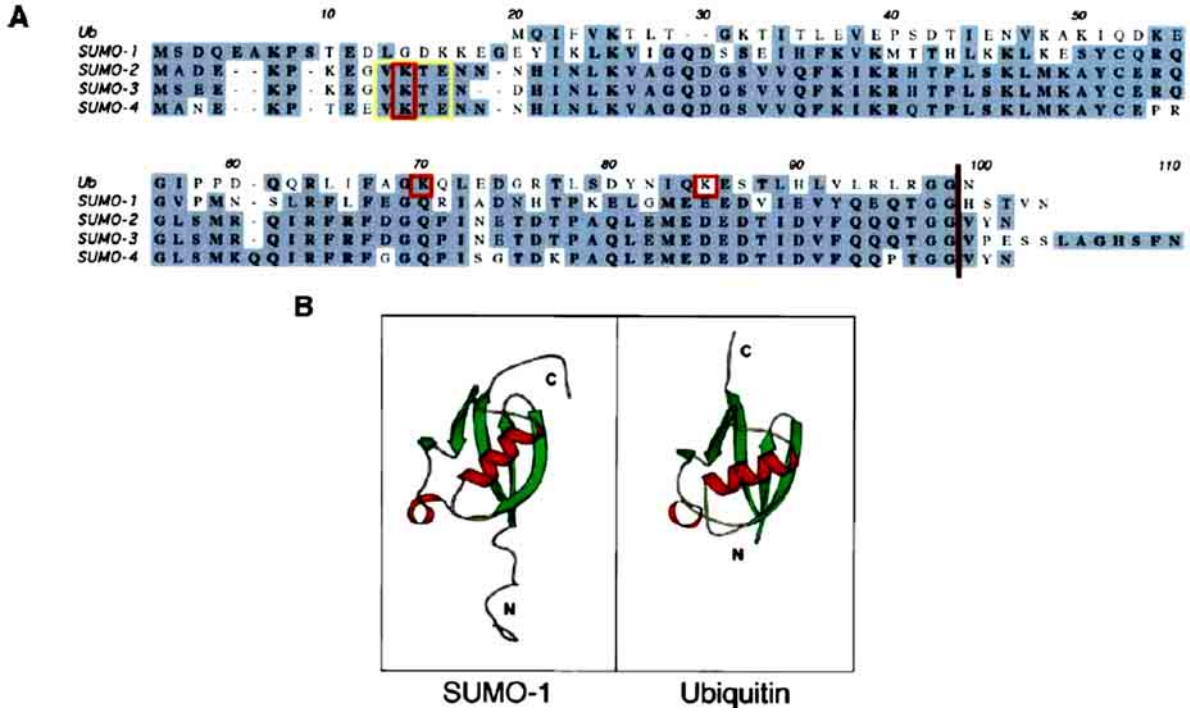


Fig.1.7. SUMO is highly related to ubiquitin.
A. Amino acid sequence alignments of ubiquitin and the four SUMO homologs from human. Identities are indicated in bold and similarities are shaded. A consensus motif for SUMOylation present in SUMO2, SUMO3, and SUMO4 is boxed in yellow; the SUMO acceptor lysine (K) in this motif is boxed in red. Ubiquitin Lys 48 and Lys 63, which serve as common sites for ubiquitin polymerization, are boxed in red. The site of cleavage to produce the mature proteins with C-terminal glycine–glycine residues is also indicated. A polymorphism at position 55 in SUMO4 (M55V) has been described in the human population.
B. Ribbon diagrams highlight the similarity of the three-dimensional structures of SUMO1 and ubiquitin. Secondary structure elements are indicated: β-sheets are green and α-helices are red. Notably, SUMO has an N-terminal extension not found in ubiquitin.
(After Gill, 2004).

In mammals, several SUMO proteases have been confirmed with the possibility of many more being present due to alternative splice variants (Melchior et al., 2003). As with yeast, many of the mammalian SUMO proteases are localized to different cellular compartments, which may function to regulate the balance of protein SUMOylation in these compartments.

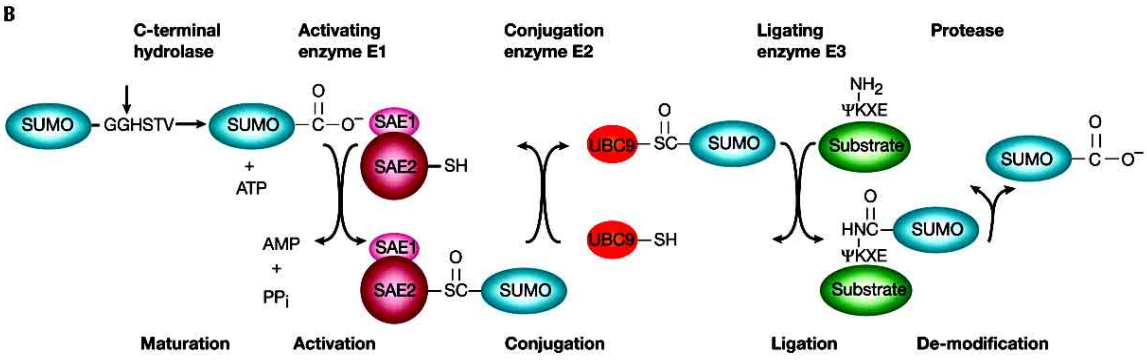


Fig.1.8. The SUMO conjugation pathway.
SUMO is synthesized as a precursor and processed by hydrolases to make the carboxy-terminal double-glycine motif available for conjugation (vertical arrow). It is subsequently conjugated to proteins by means of E1 activating (SAE1 (AOS1) / SAE2 (UBA2)), E2 conjugating (Ubc9) and E3 ligating enzymes. The E3-like proteins might serve to increase the affinity between Ubc9 (E2) and the substrates by bringing them into close proximity in catalytically favorable orientations, allowing sumoylation to occur at a maximum rate. The resulting isopeptide bond is stable and its disruption requires a desumoylating enzyme. (After Verger et al., 2002)

The thioester linked Ubc9–SUMO conjugate then catalyses formation of an isopeptide linkage between the C-terminus of SUMO and a ε-amino group of a lysine residue in the target protein.

The unusual aspect of SUMOylation is that the E1 and E2 enzymes (together with ATP and SUMO) appear to be sufficient *in vitro* for relatively robust modification of proteins at precisely the lysines that are preferred *in vivo*. For ubiquitin, an additional factor, called an E3 or ubiquitin-protein ligase, is almost always necessary for efficient substrate ubiquitination, both *in vitro* and *in vivo*.

The final step of SUMOylation also involves ligation of SUMO to the target protein. Until recently there was speculation as to whether SUMO ligation to target proteins involved E3 ligase-like proteins such as is required for ubiquitination. However, it is now clear that such E3 ligases do exist for the SUMO1 modification pathway and that they play important roles in modulating the efficiency of SUMO attachment to target proteins (Melchior et al., 2003). As with the ubiquitin system, SUMO E3 proteins are defined by 3 characteristics: binding to the substrate protein

either directly or indirectly, binding to the E2 conjugation enzyme, and ability to stimulate transfer of the modifier to the substrate or to another modifier in the case of modifier chain formation. Three different general types of SUMO E3 ligases have been described (Takahashi et al., 2001; Kotaja et al., 2002; Pichler et al., 2002). The first E3 group comprises the PIAS family of proteins. In yeast only two E3 proteins have been identified (Siz1 and Siz2) which have sequence similarity to mammalian PIAS proteins, of which at least five members have SUMO E3 activity for substrates, such as p53, Jun, Lef-1 and the nuclear androgen receptor (AR), (Seeler and Dejean, 2003).

More recently, it was shown that the nucleoporin RanBP2 (RAN-Binding Protein 2) also functions as a SUMO E3 ligase for three substrates - the promyelocytic leukemia (PML) nuclear body (NB) protein SP100, the histone deacetylase HDAC4 (Kirsh et al., 2002) and the p53 ubiquitin ligase MDM2. Although RanBP2 does not interact directly with the substrate, but possibly through Importins and Ubc9, it fulfils the above criteria for E3 ligases (Pichler et al., 2002).

A very recent report showed that the Polycomb Protein Pc2 is SUMO modified and as it enhances the modification of the transcriptional corepressors CtBP and CtBP2, can be considered as a third type of SUMO E3 ligase (Kagey et al., 2003). Given that this now makes three quite unrelated proteins that function in this capacity, it is likely that additional proteins with E3 ligase activity await discovery.

1.3.2. The Eukaryotic Family of PIAS Proteins

The eukaryotic family of protein inhibitor of activated STATs (PIAS) proteins represents a group of proteins that play a pivotal role in the control of various important cellular pathways. The acronym PIAS stems from the initial finding that members of this family act as inhibitors of STAT-transcription factors. Work by many research groups during the last few years has demonstrated that the cellular function of PIAS proteins goes far beyond inhibition of STATs.

The eukaryotic family of PIAS proteins is evolutionarily conserved from yeast to humans. In the yeast *Saccharomyces cerevisiae* two members of the family (Siz1/Siz2) are found. In higher eukaryotes the family is more diversified. The human family of

PIAS proteins consists of at least five members, PIAS1, PIAS3, the a and b splice variants of PIASx, and PIASy.

PIAS1 was isolated by Liu and coworkers (1998) from a human JY112 B cell cDNA library and by Tan and coworkers (2000) from a HeLa cell library using yeast two-hybrid screening for STAT1 and AR interacting proteins respectively. PIAS1 was shown to bind STAT1 and inhibit STAT1 binding to its consensus response element. PIAS1 inhibition of activated STAT1 signaling was demonstrated in cotransfection assays with interferon γ stimulated 293 cells using a STAT1 reporter gene (Liu et al., 1998). In an earlier study we reported that PIAS1 is a transcriptional coactivator with (AR) androgen receptor and (GR) glucocorticoid receptor but a repressor with (PR) progesterone receptor (Tan et al., 2000). PIAS1 is expressed predominantly in testis including cell types that express AR and mediate the actions of androgen on spermatogenesis. In addition to PIAS1 that inhibits STAT1, another member of the PIAS family, PIAS3 has been shown to be an inhibitor of STAT3 signaling. PIAS3 mRNA was also abundant in human testis, but unlike PIAS1, it was expressed at similar levels in other organs (Chung et al., 1997). PIASxα (ARIP3) was also isolated as an AR interacting protein by two-hybrid screening of a mouse embryo library and found to be highly expressed in rat testis (Moilanen et al., 1999).

With a length of 651-amino acid residues PIAS1 is the largest protein within this family, whereas PIASy is the smallest with 510 residues. With the exception of a variable C-terminal region the family members are highly homologous, showing an overall identity ranging from 50 to 60% at the amino acid level. Together with their orthologues from yeast or *Drosophila*, all mammalian PIAS forms share a characteristic domain structure that is schematically depicted in Fig.1.9. Within the N-terminus of PIAS a region of about 35 amino acids spans a so-called SAP module. The acronym SAP refers to three of the defining members of the class of SAP-containing proteins, scaffold attachment factor (SAF), acinus, and PIAS. The SAP domain shows a bipartite distribution of conserved hydrophobic and polar amino acids that are separated by a region containing an invariant glycine residue. Secondary structure modelling predicts that the module forms two amphiphatic helices that fold

into a hook like structure sharing significant homology with the orientation of helix 1 and helix 2 of the homeodomain. A common feature of SAP-containing proteins is their ability to bind to chromatin. In particular, the SAP module in SAF recognizes distinct A-T-rich DNA sequence known as matrix or scaffold attachment regions (MARs/SARs). MARs/SARs are operationally defined as DNA regions that are associated with the nuclear matrix, a proteinaceous meshwork, which mediates the organization of higher-order chromatin structures. MAR sequences are involved in chromatin remodelling and transcriptional regulation (Schmidt and Müller, 2003).

Another characteristic feature of Siz/PIAS proteins is the presence of a cysteine/histidine-rich domain, known as Miz-zinc finger or SP-RING domain. This domain is related to the classical zinc-binding RING motif, which is defined by the consensus sequence CX2CX(9–39)CX(1–3)HX(2–3)C/HX2CX(4–48)CX2C. The RING-finger domain characterizes a subclass of ubiquitin E3 ligases. E3s or ubiquitin-protein ligases stimulate the attachment of ubiquitin to target proteins and are largely responsible for substrate selection. An alignment of the RING finger region from the c-cbl ubiquitin ligase with the SP-RING motif from PIAS is shown in Fig.1.9. When compared with the canonical RING finger, the SP-RING motif lacks the third and sixth cysteine residues that are part of the first and third pair of cysteine/histidine residues in the RING motif. In a so-called cross-brace arrangement this first and third pair of cysteine/histidine residues forms one zinc binding site, while the second and fourth pairs form the second binding site in the RING motif.

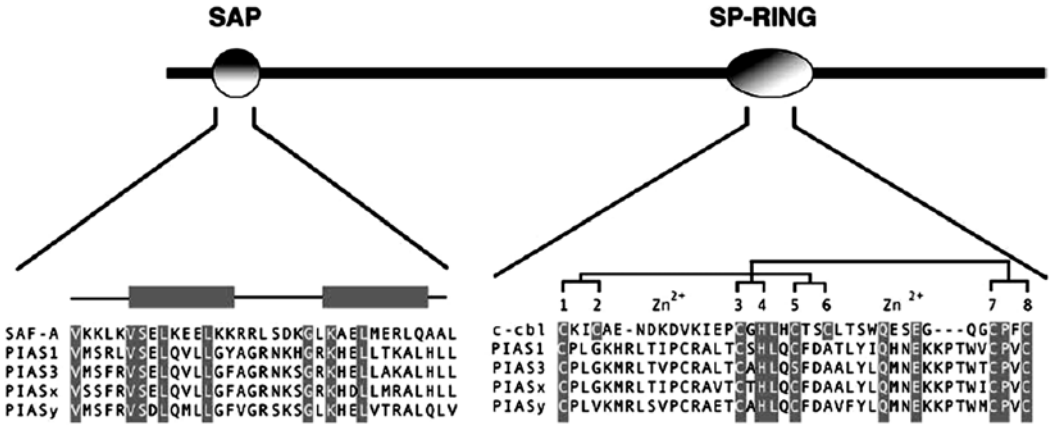


Fig.1.9. Domain structure of the Siz/PIAS family. On the left the N-terminal SAP

module from SAF-A is aligned with the SAP region from the human PIAS-family members. A schematic representation of the predicted secondary structure (two amphiphatic helices) is shown above. On the right the RING-finger region from the c-cbl ubiquitin ligase is aligned with the SP-RING motif from the human PIAS family members. The 'cross-brace' arrangement of the zinc coordinating residues in c-cbl is shown above. (After Schmidt and Müller, 2003).

The evidence concerning SUMO E3 ligases highlights another difference with the ubiquitin system. Although the SUMO E3 enzymes are able to undergo a process similar to the ubiquitin E3 'substrate-independent modification', in that PIAS proteins, RanBP2 and Pc2 are all SUMOylated themselves (Seeler and Dejean, 2003), there is no evidence that they form thioesters as do the HECT-domain ubiquitin ligases. So, like the RING-finger ubiquitin ligases, PIAS proteins, RanBP2 and Pc2 seem to function exclusively as additional platforms that bring the SUMO charged E2 to the substrate protein.

1.4. Aim of the Project

Small Ubiquitin-related Modifier (SUMO) is a protein moiety that is ligated to lysine residues in a variety of transcriptional regulators. Sp3, a member of the family of GC-box binding proteins, is one case of transcription factors to be modified by SUMO. SUMO modification of Sp3 occurs, *in vitro*, specifically at a SUMO consensus sequence (IK551EE) which has been mapped to a previously described inhibitory domain of the protein. Consequently, it was projected to determine if Sp3 is also a target *in vivo* for SUMO1 or for SUMO2/3 by employing overexpression experiments in mammalian cell lines. For this purpose, gene constructs encoding GFP-Sp3WT or a mutant thereof (GFP-Sp3-551K/R) should be co-transfected along with expression constructs coding for GFP-SUMO1 or GFP-SUMO2/3.

It was also acknowledged that three Sp3 isoforms exist, a 110-115 kDa Sp3 protein and two approximately 60-70 kDa Sp3 species. The next purpose should be finding out the complete isoforms expression pattern of Sp3 and whether Sp3 isoforms may undertake SUMOylation by improving conditions for endogenous Sp3 detection in immunoblot analysis. Additionally, subcellular localization of different isoformes should be surveyed.

SUMOylation is not a terminal modification, and instead there is an intracellular

steady state that reflects a dynamic SUMOylation–deSUMOylation process. It was necessary to perceive conditions whether Sp3 SUMOylation level could be altered. Sp3 expression level in different cell lines and in different mice organs should be investigated in conditions that preserve SUMOylation. Moreover, the level of SUMO modification of Sp3, from cells exposed to different stress conditions or drugs should be analyzed.

Depending on the target protein, SUMOylation can occur in the cytoplasm or nucleus, and this modification is involved in regulating the subcellular localization of a number of substrate proteins. Therefore, it was required to institute conditions for visualization of Sp3 in order to detect the true Sp3 localization pattern. In order to accomplish this objective, the subcellular distribution of endogenous or overexpressed Sp3 by immumofluorescence will be determinate as well as comparison in subnuclear localization with other Sp family members. In addition, it should be investigated whether, upon SUMO modification, the Sp3 is redistributed, like many other transcription factors in specific and distinct nuclear subcompartments, such as nucleoli, speckles and promyelocitic leukemia (PML) bodies.

PIAS1, previously cloned by a two-hybrid screen, does act as a SUMO E3 ligase towards Sp3 and it was demonstrated that PIAS1 strongly enhances SUMO-modification of Sp3 *in vitro*. Preliminary nuclear extract fractionation studies suggested that PIAS1 is part of (a) high molecular weight complex(es) *in vivo*. PIAS1-associated proteins might confer substrate specificity towards Sp3 and other transcription factors and/or regulate PIAS1 activity *in vivo*. Consequently, another purpose will be the cloning and subsequent characterisation of PIAS1-associated proteins. We suspect the identification of PIAS1-associated proteins to be an important step towards understanding PIAS1 protein functions and specificities *in vivo.*

As a prerequisite for the purification and identification of PIAS1-associated proteins, we will establish cell lines that express epitope-tagged PIAS1. It might not be possible to express PIAS1 ectopically using strong constitutively active promoters (CMV or ubiquitin promoters). Therefore, we want to use tetracycline-inducible promoters that can be stably integrated into already existing HeLa cell lines expressing the Tet-repressor fused to the VP16 activation domain.

The so-called tandem affinity purification (TAP) method can be employed allowing a two-step purification of appropriate tagged PIAS1. Although there is no doubt that the TAP-method allows the efficient isolation of protein complexes from *S. cerevisia*e, there remain some potential disadvantages. We thus would like to propose an

alternative method that might present some advantages over the currently favoured Calmodulin/Protein A TAP method. Important for the choice of the tags is their affinity and their specificity for the ligands used in affinity purification. The strongest non-covalent interaction known in nature is that between Biotin and Avidin or Streptavidin. Biotin is a naturally occurring co-factor for some metabolic enzymes, which is only active, when covalently attached to the enzymes. Small 15 amino acid artificial tags are known to be specifically biotinylated at a central lysine residue by the *E.coli* BirA ligase. The main goal is constructing PIAS1 expression plasmids with a C-terminal tag that can be biotinylated by the BirA ligase upon co-transfection of an appropriate expression construct. To enhance specificity, a second tag will be included in the PIAS1 vector (Calmodulin Binding Peptide or alternatively FLAG or Triple-FLAG).

A Triple-FLAG epitope might be appropriate since it has a very high affinity for commercially available anti-FLAG antibodies (200-fold higher than a single FLAG epitope). Both epitopes should contain N-terminally a recognition site for a protease (TEV, enterokinase or thrombin protease) to enable elution from the affinity matrices by proteolysis.

At first, the “Biotin system” will be tested in transient transfections. These preliminary experiments will advise us, (I) whether the tagged PIAS1 fusion protein will be biotinylated efficiently upon co-transfection of BirA ligase, (ii) whether tagged PIAS1 can be isolated efficiently with Streptavidin beads and (iii) whether the protease recognition sites are accessible. Next step will be establishing stably transfected cell lines. To avoid a constitutive over-expression of PIAS1 that might be toxic, employing inducible vectors is suggested. Tet-VP16 expressing HeLaS3 cells will be stably transfected with tetracycline-inducible expression vectors for tagged PIAS1 and BirA. Single clones that express biotinylated PIAS1 then will become the starting point for further analyses.

Prior to the purification of PIAS1 and associated proteins, we will analyse whether ectopically expressed biotinylated PIAS1 is present in (a) pre-formed complex(es) similar to endogenous PIAS1. For this, we will prepare nuclear extracts from induced HeLa Tet-OFF cells and fractionate the proteins through a Superose6 resin. Fractions will be analysed by Western blotting using Streptavidin-horseradish peroxidase (HRP) conjugates.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and Equipment

Chemicals were purchased from the following companies:

Amersham Pharmacia Biotech (Freiburg, Germany), Biomol (Hamburg, Germany), Bio-Rad (Boston, USA), Boehringer (Mannheim), Difco Laboratories (Detroit, U.S.A), Eppendorf (Hamburg, Germany), Fluka (Buchs, Switzerland), Gibco BRL (Berlin, Germany), Gilson (Villiers-le-Bel, France), Integra Biosciences (Hamburg, Germany), Invitrogen (NV Leek, The Netherlands), MBI Fermentas (St. Leon-Rot, Germany), Merck (Darmstadt, Germany), New England Biolabs (Beverly, U.S.A), Pierce (Rockford, U.S.A.), Promega (Madinson, U.S.A.), Qiagen (Hilden, Germany), Roche Diagnostics (Mannheim), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Sigma (Munich, Germany), Stratagene (Amsterdam, The Netherlands; La Jolla, U.S.A.)

Restriction enzymes and other DNA-modifying enzymes were obtained from New England Biolabs (NEB, (Frankfurt), Amersham (Braunschweig), or Invitrogen (NV Leek, The Netherlands).

In some experiments it was required to use a reagent from a specific supplier. In those cases the supplier's name is indicated.

Equipment	**Manufacturer**
Agarose gel electrophoresis chambers	Biorad, München
AutoLumat 953 luminometer	Berthold Technologies, Bad Wildbad
Cell culture incubator BBD6220	Kendro, Hanau
Clean bench HA2448GS	Kendro, Hanau
GeneAmp® PCR system 9700	Applied Bioystems, Darmstadt
Fluorescence microscope DMLB	Leica, Bensheim
Power supply units	Biorad, München
Sorvall Superspeed refrigerated centrifuge	Kendro, Hanau

Miscellaneous	Manufacturer
Bio-Rad Protein Assay	Biorad, München
DNA and protein size markers	Roche, Mannheim
Hybond ECL nitrocellulose membrane	Amersham Biosciences, Freiburg
Kodak BioMax X-ray film	Integra Biosciences, Fernwald
Sterile plastic ware for cell culture	Greiner, Germany

Kits used in this study

Kit	Manufacturer
ECL™ Plus detection system	Amersham Biosciences, Freiburg
Nucleobond® AX	Macherey-Nagel, Düren
QuikChange® Site-Directed Mutagenesis Kit	Stratagene, USA
QIAquick® PCR Purification Kit	Qiagen, Hilden
Ready To Go™ PCR Beads	Amersham Pharmacia Biotech, USA
Rapid Ligation Kit	MBI Fermentas, St. Leon-Rot

2.1.2. Mammalian and Insect Cell Lines

Mammalian cell lines were maintained at 37ºC in a humidified 5% CO_2-containing atmosphere. SL2-Cells (*Drosophila* Schneider Cells) were incubated at 25°C without necessity of CO_2-containing atmosphere.

Caco-2. An adenocarcinoma cell line derived from human (caucasian male 72 years old), colon metastasis. Morphology and growth: continuous culture, grown as monolayer, morphology epithelial-like. Culture conditions: DMEM (Dulbecco's Minimal Essential Medium) with 10% FCS (Fetal Calf Serum), 2mM L-Glutamine, 1% non-essential amino acids, Penicillin (100U/ml) and Streptomycin (100µg/ml). Split confluent cultures 1:3-1:6 using trypsin/EDTA; seed at 2-4x10^4 cells/cm^2. After thawing, cells might grow slowly.

HaCaT. A keratinocyte cell line with human origin. Morphology and growth: continuous culture, grown as monolayer, morphology epithelial-like. Culture conditions: DMEM with 10% FCS, 2mM L-Glutamine, Penicillin (100U/ml) and Streptomycin (100µg/ml). Split confluent culture 1:5-1:10 using trypsin/EDTA; seed at 2-4 x 10^4 cells/cm^2.

HeLa. The HeLa cell line was the first continuously cultured human epitheloid cell line established in 1951 from an adenocarcinoma of the cervix of a 31-year-old black female named Henrietta Lacks (Gey et al., 1952). HeLa cells are aneuploid and steroid hormone receptor negative. HeLa cells were cultured as monolayer in DMEM with 10% FCS, 2 mM L-Glutamine, Penicillin (100U/ml) and Streptomycin (100µg/ml). The cells were passaged after they reached 80% confluency. Confluent cultures were split 1:4 to 1:6 using trypsin/EDTA.

HeLa Tet-Off™ Stable Cell Lines. Express a Tet-Repressor-VP16 fusion protein. In the absence of Tetracycline, this fusion protein binds to the Tet-Operator expressed by response plasmid and activates the corresponding promoter (Yin *et al.*, 1996). Cell lines HeLa Tet-Off™ were cultured as monolayer in DMEM with 10% Tet System Approved FBS (BD Biosciences Clontech), 2 mM L-Glutamine, Penicillin (100U/ml) and Streptomycin (100µg/ml), 100 µg/ml Geneticin G-418 (selection antibiotic for HeLa Tet-Off™ Stable Cell Lines) and 2 µg/ml Tetracycline in order to keep transcription of Gene X turned "off". The cells were passaged after they reached 80% confluency. Confluent cultures were split 1:4 to 1:6 using trypsin/EDTA.

HEK293. A human embryonic kidney cell line.

Phoenix™. A human embryonic kidney based cell lines, created as helper-free retrovirus producer by transforming with adenovirus E1 and carrying a temperature sensitive T antigen.

Morphology and growth: continuous culture, grown as monolayer, morphology fibroblast. Culture conditions: DMEM with 10% FCS, 2mM L-Glutamine, Penicillin (100U/ml) and Streptomycin (100µg/ml). Split confluent cultures 1:5-1:10 using trypsin/EDTA; seed at 1-3 x 10^4 cells/cm^2.

IMR-32. A neuroblastoma cell line with human origin (caucasian male 13 months old). Morphology and growth: continuous culture, grown as monolayer, morphology

fibroblast-like and neuroblast-like. Culture conditions: RPMI 1640 with 10% FCS, 2mM L-Glutamine, 1mM Na pyruvate, 1% non-essential amino acids, Penicillin (100U/ml) and Streptomycin (100µg/ml). Split confluent cultures 1:3-1:5 using trypsin/EDTA; seed at 2-4x10^4 cells/cm^2.

Ishikawa cells were derived from a well differentiated human endometrial adenocarcinoma from a 39 year old woman. (Nishida et al., 1985). Cells were cultured as monolayer in MEM-E medium (prescription: 500 ml MEM-E, Gibco, Karlsruhe; 20 ml 7,5% $NaHCO_2$; 5 ml Penicillin/ Streptomycin (10000 IU/ml; 10000 UG/ml); 5 ml non-essential amino acids; 5 ml 200 mM L-Glutamine; 55 ml FCS). Cells were passaged after they reached 80% confluency. Confluent cultures were split 1:4 to 1:6 using trypsin/EDTA.

NIH3T3. An embryonic cell line derived from Swiss albino mouse.

Morphology and growth: Continuous culture, grown as monolayer, morphology fibroblast. Culture conditions: DMEM with 10% FCS, 2mM L-Glutamine, Penicillin (100U/ml) and Streptomycin (100µg/ml). Split confluent cultures 1:5-1:10 using trypsin/EDTA; seed at 1-3 x 10^4 cells/cm^2.

Saos-2. An osteosarcoma cell line derived from human (caucasian female 11 years old), bone metastasis. Morphology and growth: continuous culture, grown as monolayer, morphology epithelial. Culture conditions: DMEM with 10% FCS, 2mM L-Glutamine, Penicillin (100U/ml) and Streptomycin (100µg/ml). Split confluent cultures 1:3-1:6 using trypsin/EDTA.

SW620. An adenocarcinoma cell line derived from human (caucasian male 51 years old), colon metastasis. Morphology and growth: continuous culture, grown as monolayer, morphology epithelial-like. Culture conditions: DMEM with 10% FCS, 2mM L-Glutamine, Penicillin (100U/ml) and Streptomycin (100µg/ml). Split confluent culture 1:5-1:10 using trypsin/EDTA; seed at 2-4 x 10^4 cells/cm^2.

Wild type , Sp1- and Sp3- deficient ES (embryonic stem) cells (Bouwman et al., 2000; Göllner et al., 2001), were maintained in culture in ES medium. At 15 min before plating the ES cells on 9 cm diameter cell culture Petri dishes were treated with 1% sterile Gelatine solution. In order to prevent diffrentiation, LIF (Leukemia inhibitory

factor) was added to medium.

Culture conditions ES medium; When cells reaches 60% confluiency, split confluent cultures 1:3-1:5 using trypsin/EDTA.

ES cells -medium:	**Final concentration**	**50 ml**
10xDMEM (with 0,45% Glucose)	1x	5 ml
Penicilline/Streptomycine (100x)	1x	0,5 ml
L-Glutamin (100x)	1x	0,5 ml
Fetal Bovine Serum (FBS)	15%	7,5 ml
(embryonic stem cells qualified		
from Sigma- Aldrich)		
β-Mercaptoethanol	10^{-4}M	0,5 ml
(7µl in 10 ml 1×PBS; sterile filtered)		
Leukaemia inhibitory factor (LIF) 10^6U/ml	50 µl	1000U/ml
(ESGRO® Chemicon International, Inc., USA)		
H_2O		up to 50 ml

The pH was adjusted with 7,5% $NaHCO_3$ (steril filtered).

Wild type and Sp3-deficient mouse embryonic fibroblasts (MEF) were maintained in culture in Fibroblast medium. Split confluent culture 1:5-1:10 using trypsin/EDTA; seed at 2-4 x 10^4 cells/cm^2.

Fibroblast medium:	**Final concentration**	**450 ml**
DMEM with Glutamax-I	44,4%	200 ml
Ham's F 10	44,4%	200 ml
FCS	10%	45 ml
Penicillin/Streptomycin (100x)	1,1x	5 ml

SL2- Cells. *Drosophila* Schneider Cell line 2, (Schneider, 1972).

Cells were cultured as monolayer in Schneider *Drosophila* Medium. (For 500 ml *Drosophila* Schneider medium (Life Technologies, Inc.) add 50 ml (10%) fetal calf serum (insect cell qualified; Life Technologies, Inc.), 5 ml Penicillin/ Streptomycin (10000 IU/ml; 10000 UG/ml), Gibco, Karlsruhe, 5 ml 200 mM L-Glutamine, Gibco, Karlsruhe. Incubate the SL2- Cells at 25°C without CO_2-containing atmosphere requirement.

2.1.3. Buffers and Solutions

6×Gel-loading Buffer: 0,25% (w/v) bromophenol blue; 0,25% (w/v) Xylen-Cyanol; 30% (v/v) glycerol in H_2O.

PBS (pH 7.4): Dissolve 8 g of NaCl; 0,2 g of KCl; 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml of distilled H_2O. Adjust the pH to 7,4 with HCl. Add H_2O to 1 liter. Sterilize them by autoclaving and store at room temperature. The final concentrations of the ingredients are 137 mM NaCl; 2,7 mM KCl; 4,3 mM Na_2HPO_4; 1,4 mM KH_2PO_4.

TE (pH 8.0): 10 mM Tris (pH 8); 1 mM EDTA (pH 8).

6×SDS Gel-loading buffer: 280 mM Tris-Cl (pH 6.8), 12 % (v/v) SDS, 60 % (v/v) glycerol, 0.25% bromophenol blue.

TAE (pH 8.0): 40 mM Tris-acetate; 2 mM EDTA (pH 8).

TBE: 90 mM Tris; 90 mM boric acid; 2 mM EDTA.

10×SDS electrophoresis buffer: 1,92 M glycine; 0,25 M Tris base; 1% SDS.

Tris-glycine SDS buffer: 25 mM Tris-Cl; 250 mM glycine; 0,1% SDS.

2.1.4. Enzymes and Antibodies

All restriction endonucleases, T4 DNA ligase, T4 DNA polymerase and Klenow fragment were supplied by Gibco Invitrogen, New England Biolabs, Roche and Boehringer.

The following antibodies were used:

-Rat **anti-HA** High Affinity antibody (3F10) recognizes the HA peptide sequence [YPYDVPDYA] derived from the human hemagglutinin protein. (Roche Diagnostics GmbH, Mannheim).

-Rabbit **HA-probe** (Y-11). Polyclonal IgG – (Santa Cruz Biotechnology).

-Mouse **anti-FLAG** (M2, Sigma).

-Living Colors (**anti-GFP**) Monoclonal Antibody (JL-8) - (Clontech).

-**Anti PIAS 1** (C-20): *sc-8152* is an affinity purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of PIAS 1 of human origin (differs from corresponding mouse sequence by a single amino acid) (Santa Cruz Biotechnology).

-Rabbit polyclonal **anti Sp1** and **anti Sp3** antibodies (Hagen et al., 1994).

-**Anti Sp3** (D-20) is provided as a rabbit (sc-644) affinity purified polyclonal antibody raised against a peptide mapping at the carboxy terminus of Sp3 of human

origin from Santa Cruz Biotechnology, Inc USA.

-**Anti SUMO1** (FL-101): sc-4273 WB Rabbit polyclonal IgG - (Santa Cruz Biotechnology).

-Mouse **anti-GMP-1**(**SUMO1**). This monoclonal antibody can be used to specifically detect the unconjugated (~17 kDa) form of GMP1, as well as proteins covalently ligated to GMP1 (e.g. RanGAP-1) (Zymed Laboratories Inc).

Secondary antibodies used for immunofluorescence:

-**Cy3** Conjugated Affinipure goat anti rabbit (GAR) IgG (H+L)- (Jackson Immunoresearch).

-Fluorescein (**FITC**) Conjugated Affinipure GAR IgG (H+L)- (Jackson Immunoresearch).

-**Alexa Fluor 568** Conjugated Affinipure GAR IgG (H+L) – (Molecular Probes).

-**Texas Red** Conjugated Affinipure goat anti mouse GAM IgG (H+L) - (Jackson Immunoresearch).

The concentrations suggested by the suppliers are only starting recommendations. Optimal concentrations of the antibodies were determined "empirically" for each specific application.

2.1.5. Oligonucleotides

The oligonucleotides used for PCR, cloning, subcloning and mutagenesis are listed below. Oligonucleotides or were supplied by MWG-Biotech and Eurogentec.

CBPtev-for:

5'-ATTAGTCGACTCCATGGAAAAGAGAAGA-3'

CBPtev ALAspacer-rev:

5'-ATATCCGCGGGGCTTCATCGTGTTGCGCAAGAGCAGCGGTT-3'

CBP-NOT-FOR:

5'-ATATGCGGCCGCTCCATGGAAAAGAGAAGA-3'

CBP-CLA-REV:

5'-ATATATCGATTTATTCGTGCCATTCGAT-3'

Sp3-AUG-T:

5’-TAAACGAATTCTATGGCTGCCTTGGACGTG -3’

Sp3-STOP:

5’-TATTAAGGATCCCTCCATTGTCTCATTTCC-3’

NEU 5'-Sp3 I:

5’- ACTCGGAATTCCCTTTTGTGTTTCCCGCACAGTCA-3’

Sp3newXho-FOR:

5’- TAATATCTCGAGCTATGACCGCTCCCGAAAAG-3’

Sp3- MLU-FOR:

5’- TATAACGCGTCTTTTGTGTTTCCCGCAC-3’

SP3NOT-REV:

5’- CTGCGGCGGCCGCCTCCT-3’

Sp3Sal-End-REV:

5’-TATAGTCGACCTCCATTGTCTCATTTCC-3’

PIAS1-Mlu-K 5'-3':

5’-TATTACGCGTCGCCACCATGGCGGACAGTGCGGAA-3’

PIAS1-Not-rev:

5’-AATAGCGGCCGCTGTCCAATGAAATAATGTCTGG-3’

PIAS1-EcoRI-K-5'-3':

5'-TATTGAATTCCGCCACCATGGCGGACAGTGCGGAA-3’

PIAS1-Sal-rev:

5’-TATTGTCGACGTCCAATGAAATAATGTCTGG-3’

Double strand oligos for cloning:

The oligonucleotides used for tags cloning were 5’ phosphorylated and and annealed as indicated in Fig.2.1.

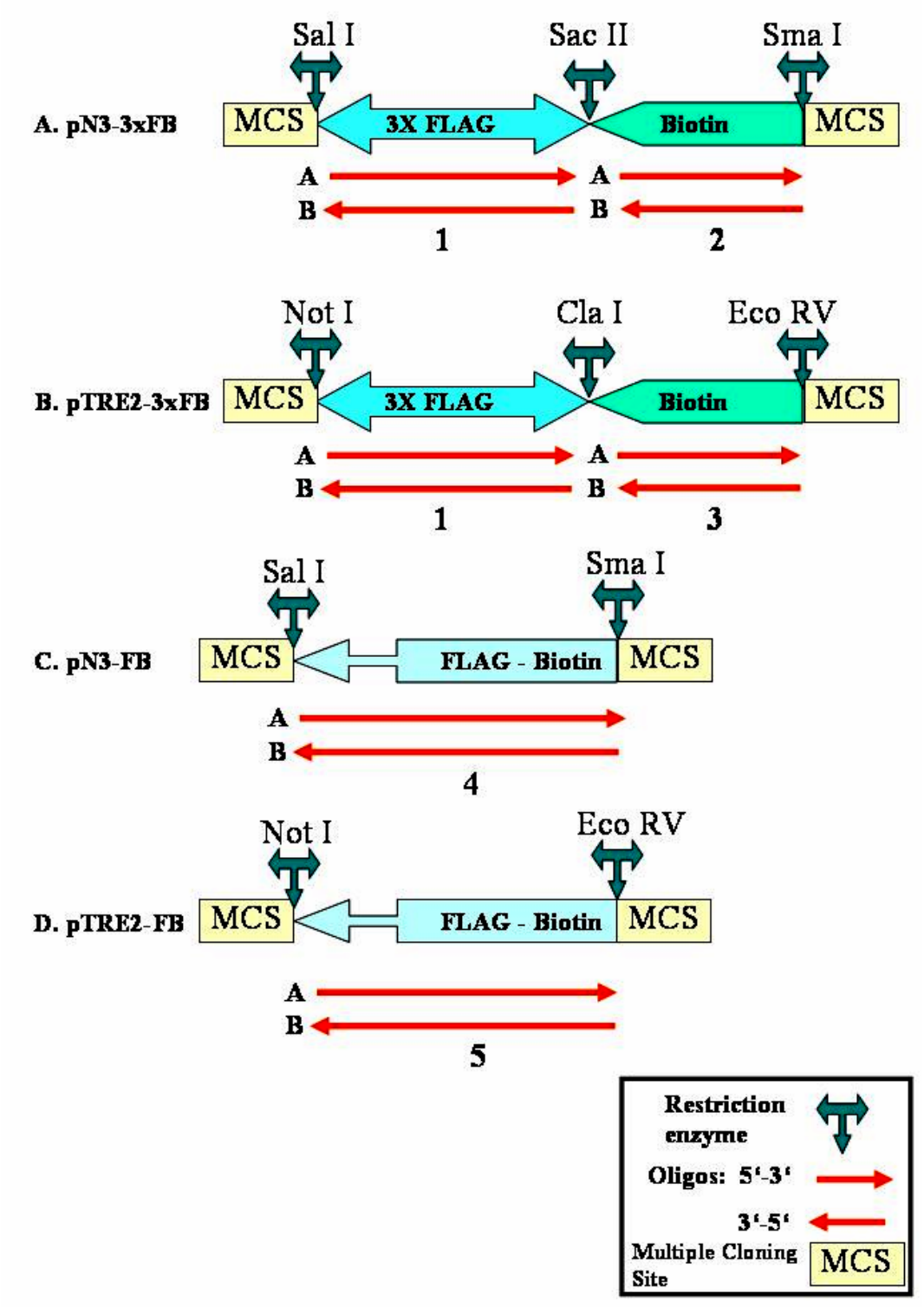


Fig.2.1. Schematic drawiong of double strand oligonucleotides and restriction sites used for construction of FLAG /3XFLAG-BiotinTAG basic vectors.

1.A. Not-Sal-3XFLAG-Sac2-Cla-5':
5’-GGCCGCGTCGACCTGGTTCCGCGTGGATCTGACTACAAAGACCATGACG
GTGATTATAAAGATCATGACATCGACTACAAGGATGACGATGACAAGCCGCG
GAAT-3’
1.B. Not-Sal-3XFLAG-Sac2-Cla-3':
5’-CGATTCCGCGGCTTGTCATCGTCATCCTTGTAGTCGATGTCATGATCTTTATA
ATCACCGTCATGGTCTTTGTAGTCAGATCCACGCGGAACCAGGTCGACGC-3’

2.A. pN3-Sac2-AviTag-Sma-5':
5’-GGGGTGGCGGTCTGAACGACATCTTCGAGGCTCAGAAAATCGAATGGCA
CGAATAACCC-3’
2.B. pN3-Sac2-AviTag-Sma-3':
5’-GGGTTATTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCAGACC
GCCACCCCGC-3’

3.A. pTRE2-Cla-AviTag-EcoV-5':
5’-CGATATGGTGGCGGTCTGAACGACATCTTCGAGGCTCAGAAAATCGAATG
GCACGAATAAGAT-3’
3.B. pTRE2-Cla-AviTag-EcoV-3':
5’-ATCTTATTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCAGACC
GCCACCATAT-3’

4.A. Sal-FlagAvi-Sma-5':
5’-TCGACGACTACAAGGATGACGATGACAAGGGTGGCGGTCTGAACGACAT
CTTCGAGGCTCAGAAAATCGAATGGCACGAATAACCC-3’
4.B. Sal-FlagAvi-Sma-3':
5’-GGGTTATTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCAGACC
GCCACCCTTGTCATCGTCATCCTTGTAGTCG-3’

5.A. Not-FlagAvi-EcoRV:5':
5’-GGCCGCGACTACAAGGATGACGATGACAAGGGTGGCGGTCTGAACGACA
TCTTCGAGGCTCAGAAAATCGAATGGCACGAATAAGAT-3’
5.B. Not-FlagAvi-EcoRV-3':
5’-ATCTTATTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCAGACC
GCCACCCTTGTCATCGTCATCCTTGTAGTCGC-3’

Oligonucleotides used for PIAS1 mutagenesis:

P1-repΔC-for
5'-ACATGACACCCATGCCTTAcGACTTACAAGGATTAGATT-3'
P1-repΔC-rev
5'-AATCTAATCCTTGTAAGTCgTAAGGCATGGGTGTCATGT-3'

2.1.6. Plasmids

Detailed descriptions of the cloning strategies were provided for my own constructs.

2.1.6.1. Expression Plasmids for Mammalian Cells

Basic vectors:

pMCS-HA (Lange et al., 2002) derivates from pEGFP-N1 (Clontech), in which the GFP part of pEGFP is replaced by the HA epitope which was cloned between *Bam*HI and *Not*I. The HA tag is in the same ORF as GFP.

pHA-MCS (Lange et al., 2002) derivates from pEGFP-C1 (Clontech), in which the GFP part of pEGFP is replaced by the HA epitope which was cloned between *Nhe*I and *Bgl*II. The HA tag is in the same ORF as GFP.

pEGFP-N3 (Fig.2.2) encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm).

Fusions to the N terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo*. The target gene was cloned into pEGFP-N3 so that it is in frame with the EGFP coding sequences. The inserted cDNA should include an ATG codon. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method. pEGFP-N3 can also be used simply to express EGFP in a cell line of interest (e.g., as a transfection marker).

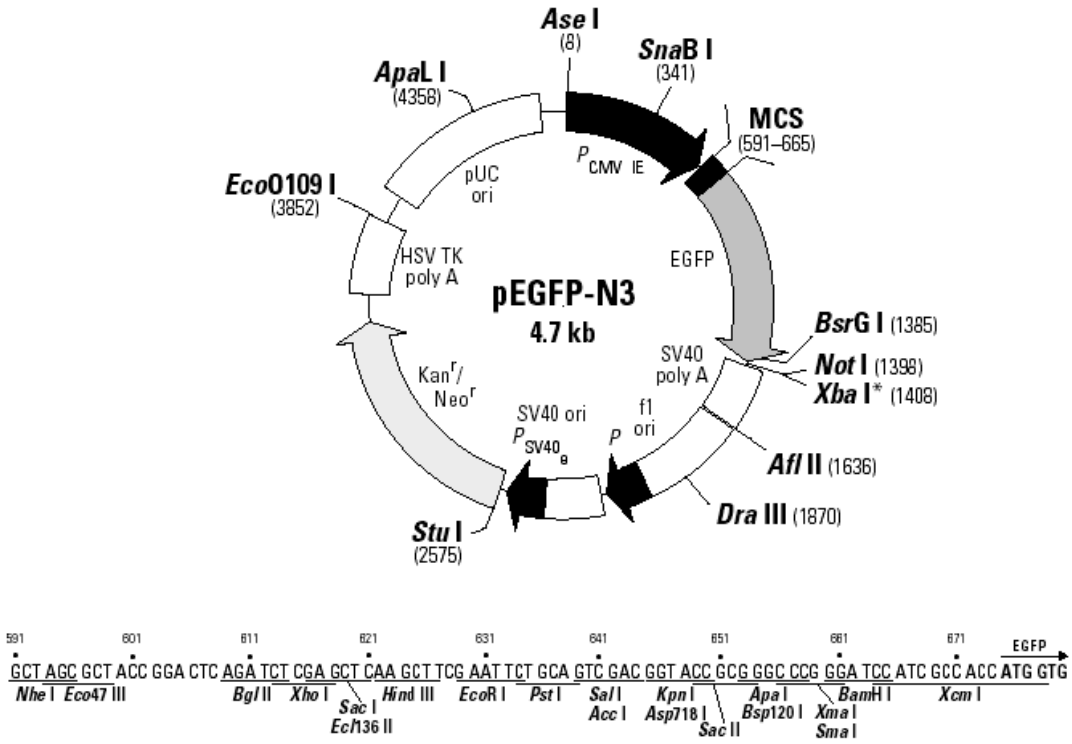
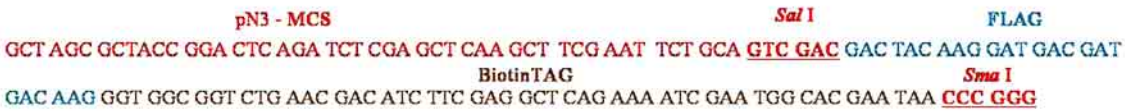


Fig.2.2. Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N3 (Unique restriction sites are in bold). The *Not* I site follows the EGFP stop codon. The *Xba* I site (*) is methylated in the DNA provided by Clontech. (pEGFP-N3 Vector Information, www.bdbiosciences.com).

pN3 was constructed by removing of the GFP moiety from pEGFP-N3 (Fig.2.2) with *Bam*HI and *Not*I followed by Klenow filling and religation.

pN3-FB. CMV-basic vector for high expression of C-terminal FLAG-BiotinTAG tagged proteins in mammalian cells. BiotinTAG can be removed by Enterokinase.



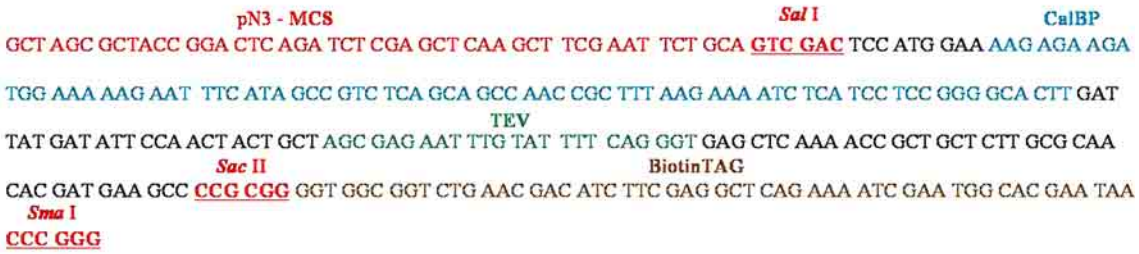
The pN3-FB was constructed by inserting of the double strand DNA tag sequence into the corresponding *Sal*I-*Sma*I -restricted pN3 plasmid (see Fig.2.1.C).

pN3-3xFB. CMV-basic vector for high expression of C-terminal triple(3x)FLAG-BiotinTAG tagged proteins in mammalian cells. BiotinTAG can be

removed by Enterokinase and 3XFLAG by Thrombin.

The pN3-3xFB was constructed by inserting of the double strand DNA tag sequences (3XFLAG-*Sal*I-*Sac*II and Biotintag-*Sac*II-*Sma*I) into the corresponding *Sal*I-*Sma*I -restricted pN3 plasmid (see Fig.2.1.A).

pN3-CTB. CMV-basic vector for high expression of the gene of interest fused with CTB (Calmodulin-TEV- BiotinTAG) tag in mammalian cells. C-terminal BiotinTAG can be removed by TEV. The CalBP-TEV fragment was insert generated by PCR using as template pBS1479 (Rigaut et al., 1999) using the following primers: sense CBPtev-for and antisense CBPtev ALAspacer-rev. The PCR product was digested with *Sal*I and *Sac*II, and cloned into the corresponding *Sal*I-*Sac*II restricted pN3 vector.



The double strand DNA tag sequence BiotinTAG- *Sac*II-*Sma*I was cloned into the corresponding *Sac*II-*Sma*I-restricted pN3 plasmid (see Fig.2.1.A for cloning).

Expression plasmids:

pEGFP-C1-Sp3fl encoding wild-type full-length Sp3 fused at N-terminal to GFP was constructed as follows. The Sp3 ORF was amplified by PCR using the following primers: sense Sp3-AUG-T and antisense Sp3-STOP. The PCR product was digested with *Eco*RI and *Bam*HI, and cloned into the corresponding *Eco*RI and *Bam*HI-restricted pEGFP-C1 plasmid (Clontech).

pEGFP-C1-Sp3fl-K/R mutant plasmid was obtained by replacing a 750 bp

*Bst*11071-*Bam*HI wild-type fragment with a corresponding fragment obtained by PCR amplification from pPacUSp3K/R plasmid, which contained the lysine to arginine mutation at the IKEE motif.

pMCS-Sp3NEW-HA

Sp3NEW insert generated by PCR using as template pN3-Sp3FLnew using the following primers: sense NEU 5'-Sp3 I and antisense SP3-STOP. The PCR product was digested with *Eco*RI and *Bam*HI, and cloned into the corresponding *Eco*RI and *Bam*HI -restricted pMCS-HA vector.

pMCS-Sp3 K/RNEW-HA

The mutant plasmid was obtained by replacing a 750bp *Bst*11071-*Bam*HI fragment from wildtype pMCS-Sp3FLn-HA by a corresponding *Bst*11071-*Bam*HI fragment from pEGFP-N3-Sp3K/R that contain the mutation in the IKEE motif.

pHA Sp3Fl* Sp3 insert was cut with *Xho*I and *Bam*HI from pEGFP-C1-Sp3fl and was cloned into the corresponding *Xho*I and *Bam*HI -restricted pHA-MCS vector.

pHA Sp3K/RFl* Sp3 insert was cut with *Xho*I and *Bam*HI from pEGFP-C1-Sp3fl-K/R and was cloned into the corresponding *Xho*I and *Bam*HI -restricted pHA-MCS vector.

pHA-Sp3NEW. The first 250 bp fragment of **pHA Sp3 Fl*** was cut with *Xho*I -*Not*I and replaced with 280bp PCR generated fragment containing additional first 13AA of Sp3new sequence by using as template: pSPT 18-Sp3new and the following primers: sense Sp3newXho-FOR and antisense SP3NOT-REV. The PCR product was digested with *Xho*I and *Not*I.

pHA-Sp3NEW K/R. The first 250 bp fragment of **pHA Sp3 K/R Fl*** was cut with *Xho*I-*Not*I I and replaced with 280bp PCR generated fragment containing additional

first 13 aa of Sp3new sequence by using pSPT 18-Sp3new as template and the following primers: sense Sp3newXho-FOR and antisense SP3NOT-REV. The PCR product was digested with *Xho*I and *Not*I.

pN3-Sp3fl* = (Δ13 Sp3). CMV-driven expression vector for full-length Sp3 (still not full-length (Sp3FL*), 13 aa at the N-term missing (second AUG)). Sp3new insert as 2600 bp fragment from pSPT18-Sp3FL* was digested *Eco*RI – *Sal*I and cloned into the corresponding *Eco*RI - *Sal*I restricted pN3 vector. Also K to R mutation in the IKEE motiv available **- pN3-Sp3 K/R fl* = (Δ13 Sp3 K/R).**

pN3-Sp3 NEW. CMW-driven expression vector for full-length Sp3 (with both upstream AUGs). Sp3new insert as 2800 bp fragment from pSPT18-Sp3FL-NEW was digested *Eco*RI - *Sal*I and cloned into the corresponding *Eco*RI - *Sal*I restricted pN3 vector. Also K to R mutation in the IKEE motiv available **- pN3-Sp3 K/R NEW**.

pN3-PIAS1-FB. CMV-driven expression vector for PIAS1-FLAG-BiotinTAG.

PIAS1wt insert generated by PCR using as template pGEX-PIAS1using the following primers: sense: PIAS1-Eco-K-5' and antisense PIAS1-Sal-rev. The PCR product was digested with *Eco*RI and *Sal*I, and cloned into the corresponding *Eco*RI and *Sal*I -restricted pN3-FB vector.

pN3-PIAS1-3xFB. CMV-driven expression vector for PIAS1-FLAG-BiotinTAG.

PIAS1wt insert generated by PCR using as template pGEX-PIAS1using the following primers: sense: PIAS1-Eco-K-5' and antisense PIAS1-Sal-rev. The PCR product was digested with *Eco*RI and *Sal*I, and cloned into the corresponding *Eco*RI and *Sal*I -restricted pN3-3xFB vector.

pN3-PIAS1-CTB. CMV-driven expression vector for PIAS1-CalBP-BiotinTAG.

PIAS1wt insert generated by PCR using as template pGEX-PIAS1using the following primers: sense: PIAS1-Eco-K-5' and antisense PIAS1-Sal-rev. The PCR

product was digested with *Eco*RI and *Sal*I, and cloned into the corresponding *Eco*RI and *Sal*I -restricted pN3-CTB vector.

pN3-Sp3-CTB. CMV-driven expression vector for Sp3new-CalBP-BiotinTAG. Sp3new insert generated by PCR using as template pMCS-Sp3new-HA using the following primers: sense: NEU 5'-Sp3 I and antisense Sp3Sal-End-REV. The PCR product was digested with *Eco*RI and *Sal*I, and cloned into the corresponding *Eco*RI and *Sal*I -restricted pN3-CTB vector.

pN3- Sp3-FB. CMV-driven expression vector for Sp3-FLAG-BiotinTAG.

Sp3new insert generated by PCR using as template pMCS-Sp3new-HA using the following primers: sense: NEU 5'-Sp3 I and antisense Sp3Sal-End-REV. The PCR product was digested with *Eco*RI and *Sal*I, and cloned into the corresponding *Eco*RI and *Sal*I -restricted pN3-FB vector.

pN3- Sp3-3xFB. CMV-driven expression vector for Sp3-FLAG-BiotinTAG.

Sp3new insert generated by PCR using as template pMCS-Sp3new-HA using the following primers: sense: NEU 5'-Sp3 I and antisense Sp3Sal-End-REV. The PCR product was digested with *Eco*RI and *Sal*I, and cloned into the corresponding *Eco*RI and *Sal*I -restricted pN3-3xFB vector.

pN3-FL-Ubc9. High level expression of FLAG-Ubc9. Obtained from Stamminger lab, Erlangen.

pN3 -FLAG-SUMO1. High level expression of FLAG-SUMO1.

pSG5LINK-EGFP-SUMO1 and **pSG5LINK-EGFP-SUMO2.** High level expression of EGFP- tagged from SUMO1and SUMO2. Obtained from Stamminger lab, Erlangen.

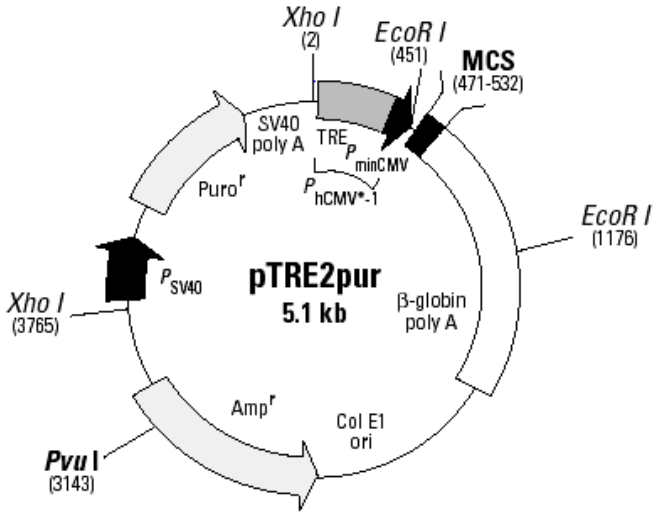
pBudCE4.1. –Bir A BirA biotin ligase (Boer et al., 2003) cloned in high level expression vector (Invitrogen). Provided by Philipsen lab., Erasmus MC, Department of Cell Biology, Rotterdam, The Netherlands.

2.1.6.2. Expression Plasmids for Generation of Stable Cell Lines:

Basic vectors:

pTRE2pur (Fig.2.3) is a response plasmid that can be used to express a gene of interest (Gene X) in Clontech's Tet-On™ and Tet-Off™ Gene Expression Systems and Tet-On and Tet-Off Cell Lines (Gossen and Bujard, 1992). pTRE2pur contains an MCS immediately downstream of the Tet-responsive PhCMV-1 promoter. cDNAs or genes inserted into the MCS will be responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems, respectively. PhCMV-1 contains the Tet response element (TRE), which consists of seven copies of the 19-bp tet operator sequence (tetO). The TRE element is just upstream of the minimal CMV promoter (Pmin CMV), which lacks the enhancer that is part of the complete CMV promoter. Consequently, PhCMV-1 is silent in the absence of binding of TetR or rTetR to the tetO sequences.

The Puromycin resistance gene is under the control of SV40 promoter and is used to directly select for stable transformants.



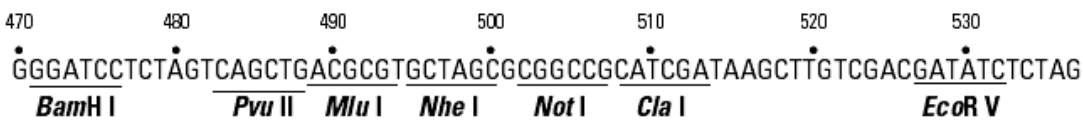
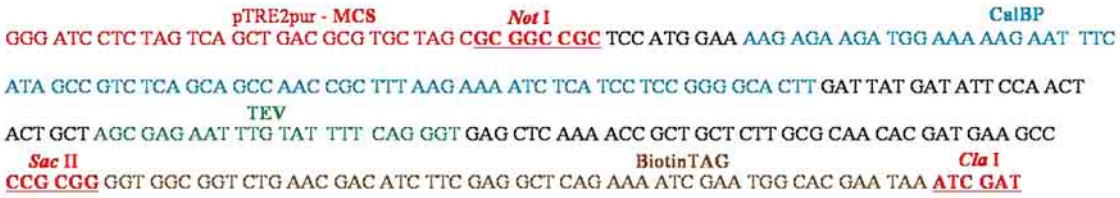


Fig.2.3. Map and Multiple Cloning Site (MCS) of pTRE2pur Vector. Unique restriction sites are in bold. (pTRE2 pur Vector Information, www.bdbiosciences.com).

pTRE2pur-Luc Control Vector, contains an additional 1649 bp encoding firefly luciferase inserted into the MCS. This vector can be used as a reporter for induction efficiency using standard luciferase detection reagents. It is not intended as a cloning vector.

pTet-Off regulatory plasmid encodes the tetracycline-controlled transactivator (tTA). In the Tet-Off System, Tet repressor (TetR), a 37-kDa protein is a fusion of amino acids 1–207 of TetR and the C-terminal 127 aa of the Herpes simplex virus VP16 activation domain. Addition of the VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). The pTet-Off regulatory plasmid also includes a neomycin-resistance gene to permit selection of stably transfected cells (see pTet-Off Vector Information, www.bdbiosciences.com).

pTRE2pur-CTB is a response plasmid that can be used to express a gene of interest fused with CTB tag in Clontech's Tet-On and Tet-Off Gene Expression Systems and Tet-On and Tet-Off Cell Lines. CalBP-TEV-BiotinTag generated by PCR using as template pN3-PIAS1-CTB using the following primers: sense: CBP-NOT-FOR and antisense CBP-CLA-REV.



The PCR product was digested with *Not*I-*Cla*I, and cloned into the corresponding *Not*I-*Cla*I -restricted pTRE2pur vector.

Expression plasmids:

pTRE2pur- PIAS1CTB is a response plasmid that expresses PIAS1fused with CTB tag in Clontech's Tet-On and Tet-Off Gene Expression Systems and Tet-On and Tet-Off Cell Lines. PIAS1-CTB PCR fragment was generated by PCR using as template pN3-PIAS1-CTB using the following primers: sense: PIAS1-Mlu-K-5' and antisense CBP-CLA-REV.

pTRE2pur-Sp3-CTB is a response plasmid that expresses Sp3 fused with CTB tag in Clontech's Tet-On and Tet-Off Gene Expression Systems and Tet-On and Tet-Off Cell Lines. Sp3 PCR fragment was generated by PCR using as template pN3-Sp3 CTB using

the following primers: sense: Sp3- MLU-FOR and antisense CBP-CLA-REV.

pBudCE4.1.-rtTA–Bir A. is a response plasmid that expresses BirA biotin ligase (Boer et al., 2003) in the tetracycline-controlled system. Provided by Philipsen lab., Erasmus MC, Department of Cell Biology, Rotterdam, The Netherlands.

2.1.6.3. Expression plasmids for insect cells:

pPac-Sp3flNEW

Drosophila expression vector for full-length Sp3 (with both upstream AUGs).

pPac-Sp3K/RflNEW

Drosophila expression vector for full-length Sp3 K/R mutant.

pPAC-Sp3-K-mut

*Drosophil*a expression vector for lysin mutants (from inhibitory domain) of Sp3 sequence. Available mutants: K to R (additiional *Eco*RI site); K to D (additional *Cla*I site); K to A (additional *Eco*RV site).

pPAC-Sp3 and pPAC-Sp3-K-D. *Drosophila* expression vector for wt and lysin mutant (K to R from inhibitory domain) of small isoforms Sp3 (Hagen et al., 1994).

2.2. Methods

2.2.1. Molecular Biological Methods

2.2.1.1. PCR

Polymerase chain reaction (PCR) was performed with with Ready To Go™ PCR Beads (Amersham Pharmacia Biotech, USA). In provided reaction tubes, 15 pmol of each primer were mixed with 50 pg template DNA and H_2O to 25 µl total volume. In principle, the PCR reactions were carried out for 30 cycles of 1 min at 94°C, 40 sec at 58°C and 1 min at 72°C, followed by an extension at 72°C for 7 min.

2.2.1.2. Purification of Nucleic Acids

2.2.1.2.1. DNA Agarose Gel Electrophoresis

2% to 0,5% agarose gels were routinely used to separate DNA fragments in a size range of 100 to 10.000 bp (Sambrook and Russell, 2001). The appropriate amount of agarose was dissolved in 1× TBE-buffer by boiling for a few minutes in a microwave oven. When the gel solution has cooled down to 60°C, ethidium bromide was added to a End concentration of 0,1 µg/ml. The clear solution was then poured into a gel mold using a suitable comb for generating the sample wells and allowed to harden for some 30-45 min. The gel was mounted in the electrophoresis chamber which was filled with 1x TBE running buffer until the gel was just submersed. DNA samples and a suitable size standard (DNA marker an *Eco*RI-*Hind*III cut λ DNA) were mixed with 0.2 volume of 6xloading buffer and applied to the wells. A voltage of 2-10V/cm was applied until the bromophenol blue had migrated an appropriate distance through the gel. After completion of electrophoresis the gel was examined on a 305 nm UV transilluminator and photographed using gel documentation system (Intas, Göttingen).

2.2.1.2.2. Electrophoretic Separation of DNA in Agarose Gels

DNA molecules were separated by horizontal agarose gel electrophoresis in TBE buffer. 0,8%-1,2% (w/v) agarose gels, containing 0,1 µg/ml ethidium bromide, were used for analysis of digested plasmids and for separation and isolation of PCR bands. DNA samples were loaded into the gels in 1 x DNA loading buffer and run at 5-10 V/cm of gel. Generally samples were run in parallel with a DNA marker. Linear fragments used for further enzymatic treatments were isolated from agarose gels after electrophoresis. Agarose pieces containing the desired DNA fragment were visualized on UV light (for short time at 254 nm in order to avoid DNA degradation) and were excised with a scalpel blade.

2.2.1.2.3. Extraction of DNA and Isolation from Agarose Gels

For separation of DNA from proteins, phenol/chloroform extraction was performed followed by ethanol precipitation washing (Sambrook *et al.*, 1989). In the final steps of vector preparation, purification of PCR products and purification of gel isolated DNA fragments, QIAquick pre-packed Micro Spin columns were used following the instructions of the supplier (QIAGEN).

2.2.1.3. Enzymatic Manipulation of DNA

2.2.1.3.1. Digestion of DNA with Restriction Endonuclease

DNA samples (200 ng–1 µg) were digested by restriction endonucleases using corresponding reaction buffers. Generally, restriction digests were prepared in 20 µl total volume, with 0.5-1 µl restriction enzyme (1-5 U/µl) and incubated at 37°C from 1 h to 3 h. In other cases, enzyme, DNA, buffer volumes and reaction times varied depending on the specific requirements. For enzyme inactivation, samples were incubated at 65°C for 10 min or were mixed with 0.2 volume of 6xloading buffer.

2.2.1.3.2. Dephosphorylation of DNA with Alkaline Phosphatase

For insertion of the DNA fragment of interest into a plasmid, a modification of the vector is required. One such modification is dephosphorylation with calf intestine phosphatase (CIP), which catalyzes the hydrolysis of the 5‘phosphate residue, thus preventing self ligation of vector termini. The reaction components were the buffer (50 mM Tris HCl pH 8.0, 1 mM $ZnCl_2$, 1 mM $MgCl_2$), 1-20 pmol DNA termini, and 0,1 U CIP. The reaction (50 µl) was performed at 37°C for 30 minutes and stopped for 10 minutes at 75°C.

The phosphatase treatment was done directly following cleavage by a restriction endonuclease. A conversion factor for calculation of amount of the DNA was that 1 µg of a 3-kb linear DNA contains 1 pmol of 5‘termini.

2.2.1.3.3. Ligation

Ligations were done by using a vector to insert ratio 3 : 1, with 200 ng vector-DNA in 20 mM Tris/HCl, pH 7,5; 10 mM MgCl2; 10 mM DTT; 1 mM ATP with 1 U T4-DNA-Ligase over night at 16°C.

Rapid Ligation Kit (MBI Fermentas) or Fast Link™ System (Biozym, Hessisch Oldendorf) were used following the instructions of the suppliers.

2.2.1.3.4. Hybridizing of Synthetic Oligonucleotides for Cloning

Equimolar amount of complementary synthetic oligonucleotides (1 µg to 5 µg of each) were incubated for 20 min at 75°C in 30 µl 10 mM Tris/HCl; 1 mM EDTA; 30 mM NaCl; pH 8. The hybridization reaction was cooled down by unplugging the incubator.

2.2.1.3.5. Site-Directed Mutagenesis

Stratagene’s QuikChange® site-directed mutagenesis kit allows site specific mutation in

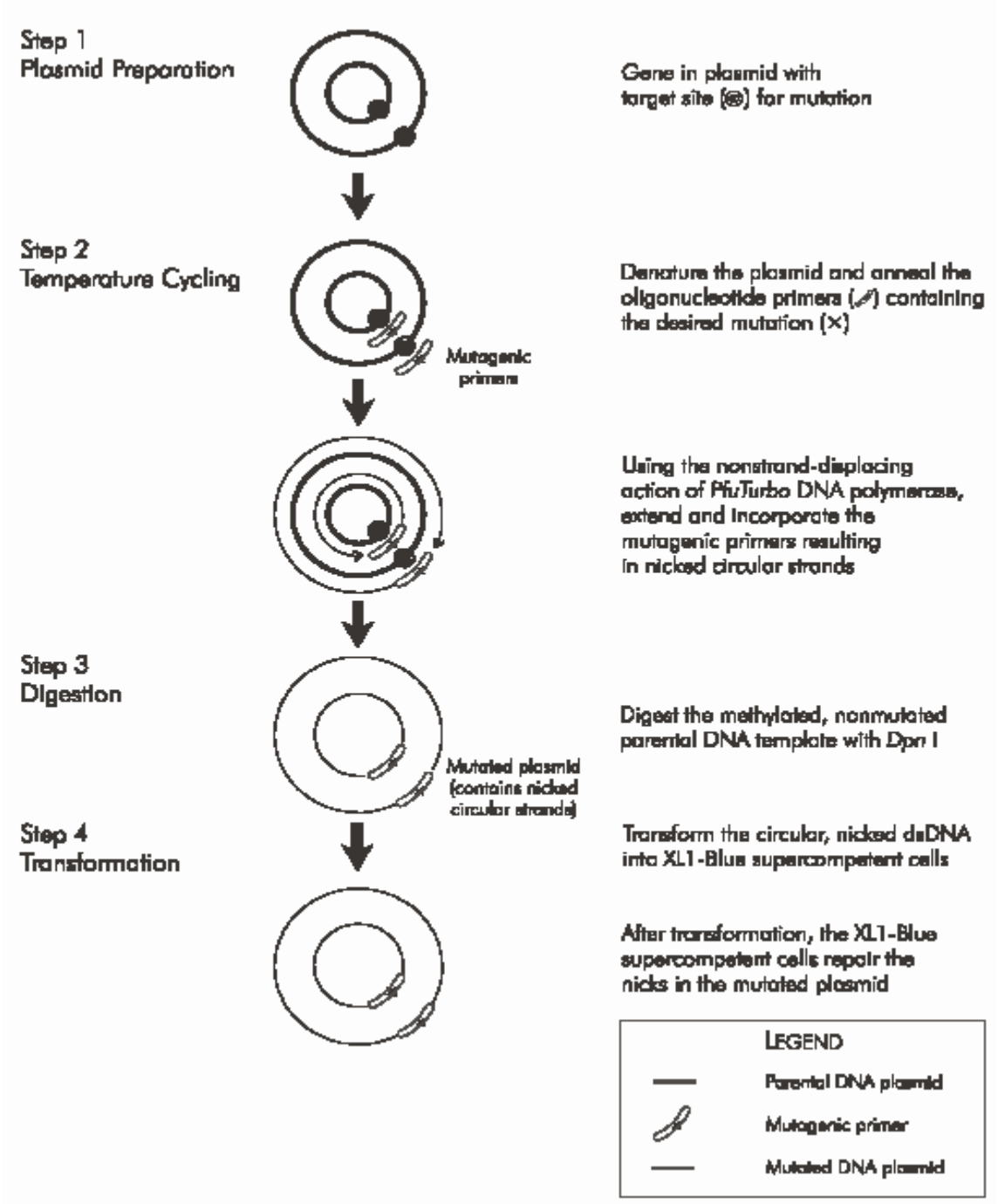


Fig.2.4. Overview of the QuikChange® XL site-directed mutagenesis method. (After Stratagene, QuikChange® XL Site-Directed Mutagenesis Kit, Instruction Manual, www.stratagene.com).

virtually any double-stranded plasmid. The QuikChange site-directed mutagenesis kit is used to insert point mutations, switch amino acids, and delete or insert single or multiple amino acids. *PfuTurbo* DNA polymerase replicates both plasmid strands with high fidelity without displacing the mutant oligonucleotide primers. The basic procedure

utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation (see Fig.2.1). The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn*I. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn*I digestion. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells.

For correcting a point mutation in PIAS1 sequence, we used as oligos: P1-repΔC-for and P1-repΔC-rev, following the manufacturer protocol, schematically illustrated in Fig 2.1.

2.2.2. Microbiological Methods

2.2.2.1. Cultivation and Growing of Microorganisms

Media for *E. coli* were prepared as previously described (Sambrook et al., 1989), and supplemented in function of each experiment, with antibiotics and necessary reagents. Ingredients were added to bidistilled H_2O water, poured into bottles with loosen caps and autoclaved at 120°C for 20 min. For solid media, 15 g agar per liter was added. Glassware and porcelain was sterilized in the heat sterilizer for 3 h at 180°C. Heat-sensitive solutions such as antibiotics, were filer-sterilized with 0.22 μm pore filter membrane (Millipore, France), and added to the media after autoclaving.

2.2.2.1.2. Growth Conditions and Storage of Transformed E. coli Strains

Cultures of transformed *E. coli* strains were overnight cultivated on LB plates with appropriate antibiotics at 37°C. Liquid culture was inoculated from a single colony and incubated in LB medium containing appropriate antibiotics at 37°C with 180 rpm overnight shaking. For storage of *E. coli* strains, freshly grown bacterial suspension was adjusted to 15% end concentration of sterile glycerol and frozen at –80°C.

2.2.2.1.3. Transformation of the Competent Cells with Plasmid DNA

E. coli strains were transformed in order to clone recombinant plasmid or to express recombinant proteins following the protocol described in Hanahan, 1983.

Preparation of competent E. coli and transformation

For preparation of competent *E. coli*, use an inoculating loop to streak *E. coli* DH 5α strain directly from a frozen stock onto the surface of an SOB agar plate. Incubate the plate for 16 hours at 37°C. Pick one colony and let it grow in 3 ml LB medium overnight, and then inoculate 30 ml SOB culture containing 20 mM $MgSO_4$ with 100 µl overnight culture. Grow the cells for 2.5-3.0 hours at 37°C, monitoring the growth of the culture each 20 minutes. When the culture reached an OD_{600} = 0,5 - 0,1, the cells were stored on ice for 10 minutes. The cells were collected at 2500 rpm for 10 minutes at 4°C in one 50 ml falcon tube. Discard the medium and stand the tubes in an inverted position to allow the last traces of medium to drain away. Resuspend in 10 ml TFB buffer and centrifuge at 2500 rpm for 10 minutes at 4°C. Discard the medium and resuspend in 2,4 ml TFB buffer. After gently mixing the buffer with 84 µl DMSO (7% v/v), the mixture was kept on ice 5 minutes, then with 84 µl 1M DTT and stored on ice for 10 minutes. Finally, 84 µl DMSO was added and after 5 minutes incubation on ice, the mixture was dispensed 200 µl aliquots into 1.5ml chilled, sterile microfuge tubes. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen. Store the competent cells at -80^{0}C until needed.

Using no more than 25 ng transforming plasmids in a volume not exceeding 3 µl for 200 µl competent cells, swirl the tubes gently to mix the DNA and bacteria. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 minutes. Transfer the tubes to a preheated 42°C heatblock. After exactly 45 seconds, rapidly transfer the tubes to an ice bath. Add 800 µl of SOC medium to each tube after 1-2 minutes cooling. Incubate the tubes for 45 minutes in the shaking incubator to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. Transfer 200 µL transformed competent cells culture onto 90 mm agar LB plate containing the appropriate antibiotic. Store the plates at room temperature until the liquid has been absorbed. The plates were inverted and incubated at 37°C overnight. The positive colony was screened by restriction enzyme analysis or direct DNA sequence analysis.

TFB buffer: 10mM KMES, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 100mM KCl, before adding $MnCl_2$ adjust pH to 6.7 with KOH

SOB medium: For 500 ml: 10g Select Peptone; 2,5g Select Yeast Extract; 0.292 g NaCl; 0,093 g KCl.

SOC medium: For 10 ml: 10 ml SOB; 100 µl $MnCl_2$ (2M); 200 µl glucose (1M).

LB Medium (Luria-Bertani Medium): For 1000 ml: 10 g Select Peptone; 10 g NaCl; 5 g Select Yeast Extract.

Antibiotics for *E. coli* media (end concentrations):

Ampicillin (Sigma, München) -100 µg/ml

Kanamycin (Sigma, München) -50 µg/ml

Zeocin™ (Cayla, France) -100 µg/ml

2.2.2.2. Plasmid DNA Preparation from *E. coli*

For isolation of plasmid or cosmid DNA (Sambrook et al., 1989) an alkali-lysis method was used. Buffers from Nucleobond® Plasmid DNA Purification Kit (Macherey-Nagel) were used. For DNA small volumes (miniprep), 1.5 ml of overnight liquid culture was centrifuged 1 min at 13000 rpm, the pellet resuspended in 100 µl S1 Buffer (50 mM Tris/HCl; 10 mM EDTA; 100 µg (A/ml) RNase, pH 8,0), then 100 µl of S2 Buffer (200 mM NaOH; 1% SDS) added and gently mixed, followed by addition of 100 µl S3 Buffer (2,8 M KAcetate; pH 5,1). After 10 min incubation in ice and 10 min centrifugation, plasmid DNA-containing supernatant was precipitated with 0.7 vol. isopropanol, followed by 70% EtOH washing. The dried pellet was resuspended in 10/1 TE buffer. For large DNA volumes (midipreps), plasmid DNA from 100 ml E. coli overnight liquid culture was extracted using a Macherey-Nagel Nucleobond® Plasmid DNA Purification Kit, according to the manufacturer protocols.

Plasmid DNA concentration was determined via absorption measurement with 260 and 280 nm in a spectrophotometer (Pharmacia LKB), with a quartz cuvette or by comparison between the intensity of ethidium bromide DNA bands on agarose gels and the intensity of defined standards.

Plasmid DNA for transfections was prepared from transformed *E. coli* DH5α using Nucleobond PC 500 columns (Macherey & Nagel, Düren). The quality of all plasmids (ratio supercoiled vs. nicked better than 70:30) was checked by agarose gel electrophoresis before transfection.

2.2.3. Working with Eukaryotic Cell Lines

Establishment and maintenance of mammalian cell cultures require a regular routine for preparation of media, and feeding, and subculturing the cells.

Cultures should be examined regularly to check for signs of contamination and to determine if the culture needs feeding or subculturing.

2.2.3.1. Trypsinizing and Passaging Cells

1. Aspirate the medium and discard.
2. Wash cells with PBS, aspirate, and discard. The volume of PBS should be approximately the same as the volume of medium used for culturing the cells.
3. Repeat step 2.
4. Add enough 1x trypsin-EDTA solution (Gibco, Neu Isenburg) to cover the monolayer, and rock the flask/dish 4–5 times to coat the monolayer.
5. Loosen the flask cap and place the flask/dish in a CO_2 incubator at 37°C for 1–2 min.
6. Remove flask/dish from incubator, tighten the flask cap, and firmly rap the side of the flask/dish with palm of hand to assist detachment.

If cells have not dislodged, loosen the flask cap and return the flask/dish to the incubator for a few minutes more.

7. Once dislodged, resuspend the cells in growth medium containing serum.

Use medium containing the same percentage of serum as used for growing the cells.

The serum inactivates the trypsin activity.

8. Gently pipet the cells up and down to disrupt cell clumps, and replate at desired density in fresh flasks/dishes.

If pipetted too vigorously, the cells will become damaged. Ensure that pipetting does not create foam.

2.2.3.2. Cell Freezing

1. Check that cells are healthy, not contaminated, and have the correct morphological characteristics.
2. Change the medium 24 h before freezing the cells.

Adherent and suspension cell cultures should not be at a high density for freezing. It is recommended to use cells in logarithmic growth phase.

3. For adherent cells, trypsinize cells, resuspend in medium containing serum, pellet cells by centrifugation at 100 rpm for 5 min, and resuspend cells in freezing medium (corresponding cell line medium with 10% DMSO).
4. Transfer 1.5 ml of cells into freezing vial (Cryotube- Gibco, Neu Isenburg). Label vials with name of cell line, date, passage number and growth medium.

5. Place freezing vials on canes and transfer to a polystyrene box (with walls approximately 15 mm thick) lined with cotton wool. Store box in a –80°C freezer overnight.

6. On the next day, quickly transfer the vials to the liquid nitrogen chamber, making sure that the vials do not begin to thaw.

2.2.3.3. Cell Thawing

1. Run tap water to 37°C and place a beaker under the tap.

2. Remove a vial of frozen cells from liquid nitrogen, and place in beaker containing 37°C water until fully thawed.

3. Wash the outside of the vial with 70% ethanol or another suitable disinfectant.

4. Prewarm a suitable amount of medium to 37°C in an appropriately sized flask or Petri dish. The size of flask or Petri dish depends on the cell type as well as the cell density.

5. Slowly pipet the cell suspension into the prewarmed medium. Mix during addition of the cells to the medium.

1. Immediate removal of DMSO may sometimes be necessary, especially for suspension cells. Following the addition of the thawed cell suspension to the prewarmed medium, the cells should be centrifuged and resuspended in fresh, prewarmed medium.

6. Incubate cells at 37°C overnight, and then change medium.

2.2.4. Transient Transfection and Transfection Methods

Transfection — delivery of foreign molecules such as DNA into eukaryotic cells — has become a powerful tool for the study and control of gene expression, for example for biochemical characterization, mutational analyses, or investigation of the effects of regulatory elements or cell growth behavior. Two principally different transfection approaches can be used - transient transfection and stable transfection (Freshney, 1993).

When cells are transiently transfected, the DNA is introduced into the nucleus of the cell but does not integrate into the chromosomes. This means that many copies of the gene of interest are present, leading to high levels of expressed protein. Transient transfection is most efficient when supercoiled plasmid DNA is used. Expression of the transfected gene can typically be analyzed within 24–96 hours after introduction of the DNA, depending on the construct used.

2.2.4.1. Calcium Phosphate

Calcium phosphate forms an insoluble precipitate with DNA, which attaches to the cell surface and is taken into the cells by endocytosis (Jordan et al., 1996). Twenty-four hours before transfection, harvest exponentially growing cells and replate them at a density of $1x10^5$ to $4x10^5$ cells/cm^2 Tissue culture flasks in fresh medium. Incubate the cultures for 20-24 hours at 37°C in a humidified incubator with an atmosphere of 5% CO_2. Change the medium 1 hour before transfection.

Prepare the calcium phosphate-DNA coprecipitate as follows: combine 250 µL of 2xCalcium Chloride with 5 µg of plasmid DNA in a sterile 5-ml plastic tube. Mix 1 volume of the 2xCalcium Chloride-DNA solution with an equal volume of 2xHeBS solution at room temperature. Mix the ingredients by vortexing for 1 minute and allow the solution to stand for another 1 minute. 0,5 ml of calcium phosphate-DNA suspension was immediately transferred into 4,5 ml medium above the cell monolayer. Rock the flasks gently to mix the medium, which will become yellow-orange and turbid. Carry out this step as quickly as possible because the efficiency of transfection declines rapidly once the DNA precipitate. Change the medium 24 hours after transfection. If morphological characteristics of cells are changed, replace the medium after 8 hours post-transfection. Assay the transfected cells for transient expression of the introduced plasmids by harvesting the cells 48 hours after transfection.

2xCalcium Chloride: 250 mM $CaCl_2$, 1 mM HEPES, pH 7.08.

2xHEPES-buffered saline (HeBS): 21 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.7 mM Na_2HPO_4, 137mM NaCl, 5 mM KCl, 6 mM dextrose, pH 7.08 (very critical).

2.2.4.2. DEAE-Dextran

Diethylaminoethyl (DEAE)-dextran is one of the older methods for introducing nucleic acids into cultured mammalian cells (Vaheri et al., 1965).

The positively charged DEAE-dextran molecule interacts with the negatively charged phosphate backbone of the nucleic acid. The DNA–DEAE-dextran complexes appear to adsorb onto the cells and are taken up by endocytosis. The advantages of this technique are its relative simplicity and reproducibility of results.

Disadvantages include cytotoxic effects and the fact that the serum in the culture medium must be temporarily reduced during the transfection procedure. In addition, the DEAE-dextran method is preferably used for transient transfection only.

DNA preparation: The indicated volumes are for 6-wells plates (35 mm diameter).

1. Mix the desired amount (5µg) of DNA in an Eppendorf tube.
2. Resuspend in 150 µl 1x TBS.
3. Add slowly 300 µl of pre-warmed DEAE-dextran solution (10 mg/ml in 1x TBS).

Transfection:

1. Wash cells twice with 1x TBS.
2. Add the DNA solution and incubate 30 min at RT.
3. Add 2 ml of complete medium and incubate the cells for 4 hours.
4. Add 2 ml of the DMSO solution (10% in 1x PBS). Incubate 1 min at RT and rinse twice with 1x PBS.
5. Add 2 ml of fresh complete medium.
6. Change the medium 8 hours after transfection.

Cells are now ready to be treated as desired the following day.

2.2.4.3. PEI

Polyethylenimine (PEI) cationic polymer was introduced in 1995 as a versatile transfection reagent. It was successfully used for gene transfer and antisense oligonucleotide delivery both in vitro and in vivo. PEI binds tightly to DNA forming small globular or toroidal complexes that enter cells by endocytosis. Being in the endosome, PEI acts as a "proton sponge," effectively buffering the endosomal interior, facilitating endosome disruption, and thus enabling the release of the complex into the cell cytoplasm.

Prepare PEI stock solution by solving 9 mg Polyethylenimine (Sigma) in 10 ml water. Vortex vigorously. Filter through 0.22µm filter.

Transfection:

1. To transfect 3 dishes of 21 cm^2 each take two 2 ml Eppendorf cups:

Cup nr.1: mix 10 µg plasmid DNA with 750 µl 150 mM NaCl (sterile filtered).

Cup nr.2: mix 100 µl PEI stock with 650 µl 150 mM NaCl.

2. Leave at RT for 10 min.
3. Transfer PEI (Cup nr.1) dropwise to the DNA solution (Cup nr.2), do not mix!
4. Leave at RT for 10 min.
5. Rinse cells with 1x PBS change the medium of the cells.
6. Mix the PEI/DNA solution by pipetting it up and down and transfer 500 µl to each culture-dish dropwise.

7.Incubate the cells for 4 hours.

8.Rinse cells with 1x PBS and change the medium of the cells.

9.Return the cells to the incubator until the time of harvesting.

2.2.4.4. FuGENE6

Plate the cells one day before experiment. The appropriate plating density for Ishikawa cells that are 50-80% confluent on the day of experiment -1,5-2 x 10^5 cells in 4 ml medium in a 60 mm culture dish.

Preparation of the complex:

1. In a 1.5ml sterile tube add 200 µl serum free medium as diluant for FuGENE6 transfection reagent (Roche Applied Science). Add 15 µl of FuGENE6 directly into this medium. Tap gently to mix.

2. Add 3 µg DNA solution (0,5 µg/µl) to the prediluted transfection reagent. Gently tap the tube to mix the contents.

3. Incubate for 30 min at room temperature.

4. While the complex-formation takes place, gently aspirate the growth medium from the plate, and wash the cells once with PBS. Add 4 ml fresh growth medium (can contain serum and antibiotics) to the cells.

5. Dropwise, add the complex mixture to the cells, distributing it around the well. Swirl the wells to ensure even dispersal.

Return the cells to the incubator until the time of harvesting.

2.2.4.5. Effectene

The following protocol is for transfection of adherent cells in 60 mm dishes with Effectene Transfection Reagent (Qiagen GmbH, Hilden).

1. The day before transfection, seed 2–8 x 10^5 cells (depending on the cell type) per 60 mm dish in 5 ml appropriate growth medium containing serum and antibiotics.

2. Incubate the cells under their normal growth. The dishes should be 60–80% confluent on the day of transfection.

3. The day of transfection, dilute 3 µg DNA dissolved in TE pH 8 with the DNA-condensation buffer, Buffer EC, to a total volume of 200 µl. Add 20 µl Enhancer and mix by vortexing for 1 second.

4. Incubate at room temperature (15–25°C) for 2–5 min then spin down the mixture for a few seconds to remove drops from the top of the tube.

5. Add 50 µl Effectene Transfection Reagent to the DNA-Enhancer mixture. Mix by pipetting up and down 5 times, or by vortexing for 10 seconds.

6. Incubate the samples for 5–10 min at room temperature (15–25°C) to allow transfection-complex formation.

7. While complex formation takes place, gently aspirate the growth medium from the plate, and wash cells once with 4 ml PBS. Add 4 ml fresh growth medium (can contain serum and antibiotics) to the cells.

8. Add 1 ml growth medium (can contain serum and antibiotics) to the tube containing the transfection complexes. Mix by pipetting up and down twice, and immediately add the transfection complexes drop-wise onto the cells in the 60 mm dishes. Gently swirl the dish to ensure uniform distribution of the transfection complexes.

9. Incubate the cells with the transfection complexes under their normal growth conditions for an appropriate time for expression of the transfected gene. The incubation time is determined by the assay and gene used.

In many cases, removal of transfection complexes is not necessary. However, if cytotoxicity is observed, remove the Effectene–DNA complexes after 6–18 h, wash the cells once with PBS, and add 5 ml fresh growth medium.

2.2.4.6. Lipofectin

The protocol is designed for the transient or stable transfection of adherent cells in 24-well plates, with Lipofectin reagent (Gibco/BRL).

1. The day before transfection, trypsinize and count the cells, plating them at 1-3 × 105 cells per well so that they are 40-65% confluent on the day of transfection. Cells are plated in 5 ml of their normal growth medium containing serum.

2. For each well of cells to be transfected, dilute 1 to 3 µg of DNA into 100 µl of medium without serum. This can be prepared in bulk for multiple wells.

Some serum-free media formulations can inhibit cationic lipid-mediated transfection. Test media for compatibility with transfection reagent before use.

3. For each well of cells, dilute 2-20 µl of Lipofectin Reagent into 100 µl medium without serum and incubate for 30 min at room temperature.

4. Combine the diluted DNA (from step 2) with the diluted Lipofectin Reagent (from step 3). Incubate at room temperature for 20 min to allow DNA- Lipofectin Reagent complexes to form. The solution may appear cloudy but this will not impede the transfection.

5. Add the DNA- Lipofectin Reagent complexes directly to each well and mix gently by rocking the plate back and forth.

6. Incubate the cells at 37°C in a CO_2 incubator for a total of 24-48 h until they are ready to assay for transgene expression. It is not necessary to remove the complexes or change the medium. Alternatively, growth medium may be replaced after 4-6 h without loss in transfection activity.

7. A similar procedure can be used to transfect DNA for stable expression. At 24 h after transfection, passage the cells at a 1:10 or higher dilution into fresh growth medium. The next day, add selective medium for the antibiotic resistance gene transfected.

2.2.5. Stable Transfection

With stable or permanent transfection, the transfected DNA is either integrated into the chromosomal DNA. Cells that have successfully integrated the DNA can be distinguished by using selectable markers. In some cases, stable transfection causes a morphological change which can be used as a selectable trait (Spector et al., 1998).

To obtain a stably transfected homogenous cell population, a selectable marker gene (usually a eukaryotic antibiotic resistance gene) is transfected into cells together with the gene of interest. Approximately one of 10^4 cells will stably integrate DNA, although the efficiency varies with cell type.

Preceding starting stable transfection experiments with HeLa Tet-Off cell lines, the induction of Tet regulatory plasmid in the absence of Tetracycline was analyzed by monitoring of Luciferase intensity after transfecting the pTRE2pur-Luc Control Vector, used as a reporter of induction efficiency. HeLa Tet-Off cell were grown in the presence of 2 µg/ml Tetracycline in order to keep transcription of Gene X turned “off”.

2.2.5.1. Establishing a Kill Curve (Dose-Response Curve)

Prior to using G418, Puromycin and Zeocin to establish or maintain stable and double-stable cell lines, it is important to titrate with the selection agent (antibiotic) to determine the optimal concentration for selection with the particular host cell line being tested. It is also acknowledged that the kill curve can be influenced by cell density.

Therefore, it is recommended performing two experiments for each drug: (1) a titration to determine the optimal drug concentration, and (2) an experiment to determine the optimal plating density.

1. Titrate at fixed cell density.

A. Plate 2 x 10^5 cells in each of six 10-cm tissue culture dishes containing 10 ml of the HeLa medium plus varying amounts (0, 50, 100, 200, 400, 800 µg/ml) of G418. For Puromycin, add the drug at 0, 1, 2.5, 5, 7.5, and 10 µg/ml. Zeocin was tested at 0, 10, 25, 50, 100, 200, 400 and 500 µg/ml).

B. Incubate the cells for 10–14 days, replacing the selective medium every four days (or more often if necessary).

C. Examine the dishes for viable cells every day.

For selecting the optimal concentration of drug, it is suggested to use the lowest concentration that begins to give massive cell death in ~5 days and kills all the cells within two weeks. For HeLa Tet-OFF cells, I have found 400 µg/ml of G418 to be optimal, the optimal level of Puromycin was typically around 1 µg /ml and for Zeocin the level was 80 µg /ml.

2. Determine optimal plating density.

Once you have determined the optimal drug concentration, determine the optimal plating density by plating cells at several different densities in the presence of a constant amount of drug. If cells are plated at too high a density, they will reach confluency before the selection takes effect. Optimal plating density is dependent on population doubling time and cell surface area. For example, large cells that double rapidly have a lower optimal plating density than small cells that double slowly.

A. Plate cells at several different densities in each of six 10-cm tissue culture dishes containing 10 ml of the appropriate selective medium.

Suggested densities (cells/10-cm dish): 5 x 10^6, 1 x 10^6, 5 x 10^5, 2 x 10^5, 1 x 10^5, and 5 x 10^4.

B. Incubate the cells for 5–14 days, replacing the selective medium every four days.

C. Examine the dishes for viable cells every two days.

For selecting the optimal plating density, use a plating density that allows the cells to reach ~80% confluency before massive cell death begins (at about day 5). This is the cell density at which cells should be plated for selection of stable transfectants. For HeLa cells, we have found 2 x 10^5 cells/10-cm dish to be a good plating density.

Selectable markers:

G418 (for selection of Tet-Off Cell Lines). G418 is available in powdered form BD Biosciences Clontech. The effective weight is about 0.7 g per gram of powder. Make a 10 mg/ml stock solution by dissolving 1 g of powder in approximately 70 ml of DMEM (without supplements). Filter sterilize and store at 4°C. Maintenance (HeLa cells): 400

μg/ml.

Puromycin (for selection of double-stable Tet-Off cells) Available from BD Biosciences Clontech. Maintenance (HeLa cells):1 μg/ml; Selection 0.5–5 μg/ml.

Zeocin™ is the commercial name of a special formulation containing Phleomycin is available form Cayla, Toulouse France. This antibiotic of the bleomycin family is particularly useful for identification and selection of a variety of cell types harboring vectors carrying Zeocin™ resistance genes. Maintenance: 80 μg/ml; Selection 100–500 μg/ml.

2.2.5.2. Stably Transfection and Selection of Double-Stable Cell Lines

The next step is to stably transfect the stable HeLa Tet-Off cells line with the pTRE-Gene X construct. The goal is to generate a cell line that gives low background and high expression of Gene X when tested in Western blot analysis. Both expression levels and induction of Gene X can be profoundly affected by the site of integration. Insertion near an enhancer may result in high basal expression of Gene X, whereas other insertion sites may result in suboptimal induction. To find the clone with the highest induction and lowest background, it is recommend growing and analyzing as many clones as possible. In general, test at least 15 clones.

1. Grow the HeLa Tet-Off cells to 60-80% confluency in 10 cm culture dishes, each containing 10 ml of the DMEM complete medium.

2. Transfect cells with pTRE2pur-Gene X (PIAS1 or SP3) by FuGene6 or calcium phosphate method.

3. Replace medium with fresh DMEM complete medium containing the selection antibiotic (1 μg/ml Puromycin) every four days. Fresh Tetracycline must be added every two days for Tet-Off cells.

4. After about five days, cells should start to die. Split cells if they reach confluency before massive cell death begins. After 2–4 weeks, the Puromycin -resistant colonies will begin to appear.

5. Using cloning cylinders or cloning discs isolate large, healthy colonies and transfer them to 3 cm diameter multiwell plates for further propagation in selective medium. Isolate as many clones as possible.

6. Once you have developed a suitable double-stable Tet-Off cell line, prepare frozen aliquots to ensure a renewable source of the cells.

7. When expression constructs for biotin ligase (pBUD-BirA or pBUD-tTA-BirA) were cotransfected at step 2 with pTRE2pur-Gene X (PIAS1 or SP3), the medium

containing the selection antibiotic for pTRE2pur-Gene X (1 µg/ml Puromycin) is supplemented with the selection antibiotic for biotin ligase (80 µg/ml Zeocin).

2.2.6. Luciferase Assay

1. Wash cells with ice-cold PBS three times (to remove all Ca which inhibits luciferin if cells were transfected by calcium phosphate method).
2. Add 100 µl Cell lysis buffer to the cell pellet from each 35mm dish (350 µl for a 60mm dish)
3. Transfer the lysate in an Eppendorf tube.
4. Spin at 12000 rpm, 4^0C for 10 min.
5. Luciferase assay was performed by adding 100 µL of cell lysate into individual luminometer tubes containing 360 µL of luciferase assay buffer on ice. Place the tube in the luminometer. Light was measured with an AutoLumat 953 luminometer (Berthold) by injecting 100 µL of 0.2 mM luciferin solution into the sample tubes and measuring light output over 10 sec (Brasier et al., 1989).

The concentration of total protein in the lysate was determined by the Bradford assay.

Buffers:

Cell lysis buffer: 1%(v/v) Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA and 1 mM DTT.

Luciferin solution: For 2,5 ml: 500 µl of 1 mM Luciferin, 1 ml of 50 mM glycylglycine, 1 ml bidistilled H2O, 5 µl of 1M DTT.

Luciferase assay buffer: 25 mM glycylglycine, pH 7.8, 15 mM potassium phosphate, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP and 1 mM DTT

2.2.7. Biochemical Methods

2.2.7.1. Isolation of Proteins from Eukaryotic Cells

2.2.7.1.1. High salt nuclear extract preparation by high salt extraction method

For the immunoprecipitation and Western-Blot analysis.

Procedure:

1. Aspirate old media from 90 mm plate of adherent cells.
2. Wash with PBS.

3. Add dropwise 1 ml ice-cold PBS and swirl the plate.
4. Scrape the cells and transfer in 1.5ml Eppendorf tubes.
5. Centrifuge 10 sec, 13000rpm.
6. Aspirate the supernatant and resuspend the pellet in 400 µl Buffer B.
7. Incubate in ice for 10 min.
8. Centrifuge 10 sec, 13000rpm.
9. Aspirate the supernatant and resuspend the pellet in 50 µl Buffer C.
10. Incubate in ice for 20 min.
11. Centrifuge 2 min, 13000rpm.
12. Aliquot the supernatant (nuclear extract) in new 1.5ml Eppendorf tubes.

Proteinase inhibitors PMSF (Phenylmethylsulfonylfluorid) and PIC (Protease Inhibitor Cocktail (Roche, Mannheim) were fresh added to the Buffer B und C, along with DTT. The purpose of the DTT (Dithiothreitol) is to prevent aggregation due to disulfide formation between newly exposed cysteines. Fresh DTT should be present through the initial steps of the nuclear extract preparation and immunoprecipitation. The reason to use fresh DTT is that it is possible that the DTT will lose its reducing power if stored at a dilute concentration.

10 mM N-ethylmaleimide (NEM) can be added to lysis buffers in order to prevent deSUMOylation. So far, I did not observe augmentation of Sp3 SUMOylation after adding NEM or phosphatase inhibitors like 1 mM Sodium orthovanadate (Na_3VO_4), 10 mM p-nitrophenylphosphate, 1 mM Sodium fluoride (NaF).

Buffer B: 10 mM HEPES/KOH pH 7.9; 1.5mM $MgCl_2$; 10 mM KCl; 0,5 mM DTT; 0.2 mM PMSF; 1x PIC;

Buffer C: 20 mM HEPES/KOH pH 7.9; 1.5mM $MgCl_2$; 420 mM NaCl; 0.2 mM EDTA; 25%Glycerin; 0,5 mM DTT; 0.2 mM PMSF; 1x PIC.

2.2.7.1.2 Total SDS Cell Extract Preparation:

For the immunoprecipitation and Western-Blot analysis.

Procedure:

1. Aspirate old media from the plate.
2. Wash with 1xPBS.
3. Add dropwise 100 µl Lysis Buffer and swirl the plate.
4. Scrape the cells and transfer in 1.5ml Eppendorf tubes.

5. Boil the lysate for 10 min.
6. Centrifuge 10 min, 13000rpm at 4^0C.
7. Aliquot the supernatant (SDS cell extract) in new 1.5ml Eppendorf tubes.

Lysis Buffer: - Mix one part Lysis Buffer I with two parts of Lysis Buffer II (ex: 1ml Lys I + 2ml Lys II)

Lysis Buffer I: 5% SDS; 150mM TRIS/Cl pH 6,7; 30% Glycerol.

Lysis Buffer II: 25mM TRIS/Cl pH 8,2; 50mM NaCl; 0,5% NP – 40; 0,1% SDS; 0,1% Na Azid.

Fresh Added: 5mM DTT; 0.2mM PMSF; 1x PIC.

2.2.7.1.3. Total SDS Cell Extracts from Mouse Organs:

Procedure:

1. Weigh fresh organs and dice into very small pieces using a clean razor blade.
2. Disrupt and homogenize tissue in 3 ml SDS Lysis Buffer per gram of tissue with a Dounce homogenizer or a Polytron device. When using a mechanical homogenizer, begin homogenization at slow speeds until the tissue is broken into smaller pieces and then increase the speed to the maximum for 45-60 seconds. Avoid the generation of excess heat or foam. Transfer homogenized tissues to new 2 ml Eppendorf tubes.
3. Boil the lysate for 10 min.
4. Centrifuge 10 min, 13000rpm at 4^0C.
5. Transfer supernatants to new 1.5ml Eppendorf tubes and centrifuge again. Pool supernatants in the same tube. The supernatant fluid is the whole-cell lysate. Sometimes a longer centrifugation is necessary to obtain a clarified lysate.
6. Aliquot and store at –80°C. Avoid freeze/thaw cycles.

2.2.7.1.4. Radioimmunoprecipitation (RIPA) Lysate of Cells:

For the immunoprecipitation and Western-Blot analysis.

Procedure:

1. Place cells on ice.
2. Wash cells with ice cold PBS to remove media.
3. Add 1 ml RIPA buffer to 100 mm plate. Scale up or down as necessary.
4. Scrape cells into RIPA buffer and transfer to small centrifuge tube.
5. Let stand on ice for 10 min, vortexing every few minute to dissolve material. Lysates can also be passed through a needle several times to ensure adequate

solubilization.

6. Centrifuge 10 min, 13000 rpm at 4^0C.

7. Aliquot the supernatant and store at –80°C or use for protein assays.

RIPA Buffer: 150 mM NaCl; 10 mM Tris, pH 7.2; 0.1% SDS; 1% Triton X-100; 1% Deoxycholate; 5 mM EDTA. Add protease inhibitors in final concentrations of: 5 mM DTT, 0.2 mM PMSF, 1x PIC.

2.2.7.1.5. Nuclear Extract Preparation for Gel Filtration

Procedure:

1. Wash the dishes once with PBS at RT.
2. Add 5 ml ice cold PBS and harvest the cells with rubber policeman. Then collect the cells to 15 ml Falcon tube and centrifuge at 1200 rpm, 4^0C for 5 min.
3. Remove supernatant and resuspend the pellet with 1 ml of hypotonic buffer.
4. Incubate in ice for 20 min.
5. Transfer the cells to a 5 ml glass Dounce homogenizer and homogenize with 20-25 strokes using pestle B.
6. Centrifuge at 2800 rpm, 4^0C for 5 min.
7. Remove supernatant and resuspend the pellet with 200 µl of Lysis Buffer.
8. Incubate in ice for 20 min.
9. Add 200 units Benzonase and incubate at 16 ^{0}C for one hour.
10. Add 100 units Benzonase and incubate at 16 ^{0}C for an additional hour.
11. Centrifuge at 13000 rpm, 4^0C for 15 min.
12. Transfer the supernatant in new 1.5ml Eppendorf tube.

Solutions:

Benzonase (Sigma) is a genetically engineered endonuclease from *Serratia marcescens*. This promiscuous endonuclease attacks and degrades all forms of DNA and RNA (single stranded, double stranded, linear and circular) and is effective over a wide range of operating conditions. It is ideal for a wide variety of applications where complete digestion of nucleic acids is desirable.

Hypotonic buffer: 10 mM HEPES, pH 7.9; 3 mM $MgCl_2$; 10 mM NaCl.

Lysis Buffer: 50 mM TRIS pH 8.0**;** 50 mM NaCl; 2 mM $MgCl_2$; 1 mM EDTA; 0,5 % (v/v) NP-40.

Add following protease inhibitors to hypotonic buffer and high salt buffer immediately before use: 5 mM DTT; 0.2 mM PMSF and 1x PIC.

2.2.7.2. Immunoprecipitation

Immunoprecipitation (IP) is a highly specific and effective technique for analytical separations of target antigens from cell lysates or culture supernatants. Immunoprecipitation involves the interaction between a protein and its specific antibody, the separation of these immune complexes with Protein G or Protein A, and the subsequent analysis by SDS-PAGE. By combining IP with other techniques, such as SDS-PAGE and immunoblotting, it can be used to detect and quantify antigens, determine relative molecular weights, monitor protein turnover and post-translational modifications, and check for enzyme activity.

The procedure can be divided into several stages:

A. Cell Lysate Preparation

Selecting cell lysis conditions is very critical and has to be optimized with regard to cell type and how the antigen is to be used. Whereas cells without cell walls (e.g. animal cells) are easily disrupted by treatment with mild detergent, other cells may need some type of mechanical shearing such as sonication or Dounce homogenization. The choice of lysis buffer is critical and dependent on the nature of the protein to be studied. Increasing the salt concentration, decreasing the detergent concentration, or changing the detergent to Triton X-100, Saponin, Digitonin, CHAPS or others are steps that can be taken to optimize conditions for immunoprecipitation. This protocol is optimized for RIPA cells lysates and high salt nuclear extracts. Total SDS cell extracts were also successfully used for IP but all procedures were performed at room temperature in order to avoid SDS precipitation.

B. Cell Lysate Preclearing

The preclearing step is incorporated into the procedure to lower the amount of non-specific contaminants in the cell lysate and to remove proteins with high affinity for Protein G or Protein A. The success of immunoprecipitation depends on the affinity of the antibody for its antigen as well as for Protein G or Protein A. In general, while polyclonal antibodies are best, purified monoclonal antibodies, ascites fluid, or hybridoma supernatant can also be used. Protein G coupled to some insoluble matrix

(e.g. Sepharose beads) binds well to most subclasses of rat immunoglobulins and mouse IgG1, while Protein A binds much better to mouse IgG2a, IgG2b, and IgG3. For the IP, a combination 1:1 of Protein G and Protein A Sepharose Fast Flow (Amersham Pharmacia Biotech, Freiburg) was used.

1. Transfer 50µl of each Protein G and A beads slurry to an Eppendorf tube and add 400 µl cold Lysis Buffer. Spin at 13000 rpm for 30 seconds and remove the Lysis Buffer. Wash one more time with 500 µl of cold Lysis Buffer. Resuspend the beads in 100 µl of cold Lysis Buffer.
2. Add this 50µl of prepared Protein G and A beads slurry and 500 µl of Cell Lysate to an Eppendorf tube and incubate on ice for 30-60 minutes.
3. Spin at 13000 rpm for 10 minutes at 4^0C and transfer the supernatant to a fresh Eppendorf. If any bead has been transferred, spin again and carefully transfer the supernatant to another fresh Eppendorf tube.

C. Immunoprecipitation

1. Add 5-10 µg of antibody to the Eppendorf tube containing the cold precleared lysate.
2. Incubate at 4^0C for 1 hour.
3. Add 50 µl of washed Protein G and A beads slurry in prechilled Lysis Buffer (prepared as instructed in Preclearing Step from above).
4. Incubate for 1 hour at 4^0C on a rocking platform or a rotator.
5. Spin the Eppendorf tube at 13000 rpm for 30 seconds at 4^0C.
6. Carefully remove supernatant completely and wash the beads 3-5 times with 500µl of Lysis Buffer. To minimize background, care should be given to remove the supernatant completely in these washes.
7. After the last wash, aspirate supernatant and add 50µl of 2X Laemmli sample buffer to bead pellet. Vortex and heat to 90-100 ^{0}C for 10 minutes.
8. Spin at 13000 rpm for 5 minutes, collect supernatant and load onto the gel.

Supernatant samples can be collected and kept frozen at this point.

2.2.7.3. Two-Step Affinity Purification

Usually proteins targeted for purification with their associated partners are modified by addition of a peptide suitable for affinity purification. Fusion of the target protein sequence and peptide tag coding sequence is done using standard DNA cloning

techniques. The recombinant gene thus created is then introduced and expressed in the cognate host. For higher eukaryotes transient or stable transfections are performed. In our laboratory, was developed a variant of the TAP tandem affinity purification method (Rigaut et al., 1999). Our method is based on two successive affinity chromatography steps of cell extracts from mammalian cells expressing tagged protein of interest (PIAS1 or Sp3). The tag fused to a target protein is composed of BiotinTAG having very high affinity for Streptavidin, a TEV protease cleavage site and calmodulin binding peptide having high affinity for calmodulin (CTB tag). The BiotinTAG becomes biotinylated upon coexpression of BirA biotin ligase.

Procedure:

1. The HEK293 nuclear extract (approx. 100 µl) containing biotinylated CTB tagged protein, was diluted 1: 10 in IPP150 buffer in the presence of 5 mM DTT; 0.2 mM PMSF and 1x PIC in 1.5 ml Eppendorf tube.
2. Incubate the diluted nuclear extract with 50 µl Streptavidin Agarose beads (Novagen, USA). Slowly rotated the tube for 1 hour at 4°C.
3. Spin the Eppendorf tube at 13000 rpm for 30 seconds at 4^0C.
4. Carefully remove supernatant completely and wash the beads 3 times with 1ml of IPP150 buffer and once with TEV cleavage buffer.
5. Incubate the Streptavidin beads for 2h at 16 ^{0}C with 5 units recombinant TEV protease (Invitrogen) in 300µl TEV cleavage buffer.
6. Spin the Eppendorf tube at 13000 rpm for 30 seconds at 4^0C.
7. Carefully transfer the supernatant in new 1.5ml Eppendorf tube and equilibrate 1:3 with Calmodulin binding buffer and 3 µl of 1 M $CaCl_2$.
8. Incubate with 50 µl calmodulin affinity resin (Stratagene) which had been washed with calmodulin binding buffer. Slowly rotated the tube for 1 hour at 4°C.
9. Spin the Eppendorf tube at 13000 rpm for 30 seconds at 4^0C.
10. Carefully remove supernatant completely and wash the beads 3 times with 1ml calmodulin wash buffer.
11. After the last wash, aspirate supernatant and add 50µl of 2X Laemmli sample buffer to bead pellet. Vortex and heat to 90-100 ^{0}C for 10 minutes.
12. Alternatively, elute in five fractions of 100 µl with calmodulin elution buffer followed by TCA precipitation.

Solutions:

IPP150: 10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1 % NP-40.

TEV cleavage buffer: 10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1 % NP-40; 0.5 mM EDTA; 1 mM DTT (freshly added).

Calmodulin binding buffer: 10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1 % NP-40; 10 mM β-Mercaptoethanol; 1 mM MgAc; 1 mM Imidazol; 3 mM $CaCl_2$.

Calmodulin wash buffer: same as calmodulin binding buffer but 1 mM $CaCl_2$.

Calmodulin elution buffer: same as calmodulin binding buffer but 2 mM EGTA instead of $CaCl_2$.

TEV Protease. Recombinant (rTEV) is a site-specific protease purified from *E. coli* by poly histidine tag. The protease can be used for the removal of affinity tags from fusion proteins. The seven-amino-acid recognition site for rTEV is Glu-Asn-Leu-Tyr-Phe-Gln-Gly with cleavage occurring between Gln and Gly. The optimal temperature for cleavage is 30°C; however, the enzyme can be used at temperatures as low as 4°C. Following digestion, TEV Protease can be removed from the reaction via the poly histidine tag sequence (by affinity chromatography).

2.2.7.4. TCA (Trichloroacetic Acid) Protein Precipitation

To concentrate proteins for analysis by SDS PAGE:

If a small amount of protein is to be precipitated (less than a few micrograms), add Insulin as a carrier protein (10 micrograms of Sigma insulin, per sample works well).

1. Add an equal volume of 20% TCA (dissolve 500g TCA into 350 ml dH2O, store at RT) to protein sample.
2. Incubate 30 min on ice.
3. Spin in microfuge at 4°C for 15 min.
4. Carefully remove all supernatant, leaving protein pellet intact. Pellet should be formed from whitish, fluffy precipitate.
5. Add 300 µl cold acetone and spin 5 min at 4°C.
6. Repeat steps 4-5 for a total of 2 acetone washes.
7. Remove supernatant and dry pellet for 5-10 min to drive off acetone.
8. Resuspend samples in 2x loading buffer and boil sample for 10 min in 95°C before loading sample onto polyacrylamide gel. If the sample color turns yellow (acidic pH), add 1-5 drops of 1M NaOH until the sample turn into blue again.

2.2.7.5. Determination of Protein Concentration

Protein concentration was determined according to Bradford (Bradford, 1976) using the Bio-Rad protein assay (Bio-Rad, München). This measurement is based upon Coomassie® Brilliant Blue G-250 dye-binding assay. Acryl-cuvettes (Sarstedt, Nümbrecht) were used for the determination of protein concentration. 200 µl Bio-Rad Protein Dye (Bio-Rad) were added to samples and standard (BSA, bovine serum albumine) (0-50 µg / 0.8 ml H2O with diluted sample isolation buffer), afterwards they were gently mixed to avoid bubbles. After 10 min, the measurement was carried out in the photometer (Pharmacia LKB) at 595 nm.

2.2.8. Western Blotting

2.2.8.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For immunodetection of the proteins a Western Blot was performed.

The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is usually proportional to the molecular weight of the polypeptide and is independent of its sequence, the mobility of protein-SDS complexes in polyacrylamide gels is inverse proportional to the size of the protein. By using markers of known size it is therefore possible to estimate the molecular weight of a protein (Rainbow-Protein-marker from Amersham, Braunschweig).

For most purposes a 6 or 8 % resolving gel was prepared. The gel solution was poured into the assembled gel mold between two glass plates separated by 1 mm thick spacers leaving some 1 cm space for the stacking gel. The gel surface was overlaid with water or n-butanol in order to prevent inhibition of polymerization by oxygen. After polymerization was complete (30 min) the stacking gel (always 5%) was poured on top of the resolving gel, and the comb was inserted.

Samples were prepared in 1xSDS gel-loading buffer by means of a 2x concentrated stock solution. After having added 5% (v/v) β-Mercaptoethanol or 10% (v/v) 1 M DTT all samples were boiled for 3 min to denature the proteins. Both electrode reservoirs were filled with SDS electrophoresis buffer, the wells were cleaned and samples loaded. Electrophoresis was performed at 25 mA constant power until the bromophenol blue dye had reached the bottom of the gel.

2.2.8.2. Blotting

Semidry-Blot was performed with an “Fastblot-apparatus” (Biometra, Göttingen).

1. Shortly before the gel is finished running, cut the following:

One piece of Immobilon (PVDF) the size of the gel to be blotted. Exclude the stacker area and any side(s) not used.

Six pieces of Whatman paper slightly larger (1 mm) than the membrane.

2. Wet the PVDF membrane with methanol for 15 seconds. Rinse with distilled water for 2 min then wet the membrane with Anode buffer 2.

Wet two pieces of blotting paper with Anode buffer 1; one piece with Anode buffer 2 and the last three pieces of Whatman paper with Cathode buffer.

3. When the electrophoresis is finished, discard the buffer and remove the sandwich from the rig.

4. Remove side spacers and with a spatula or razor blade "pop" the plates apart and allow the gel to fall onto one of the plates. Cut off the stacker and discard. Trim sides if necessary. Incubate the gel with Cathode buffer for 5 minutes.

5. Carefully assembly the “sandwich” without trapping any bubbles between the membrane blotting papers and gel.

6. Place the two blotting papers wetted with Anode buffer 1on the bottom plate of the transfer unit. Place the remaining pieces of blotting on following order: blotting papers wetted with Anode buffer 2, the membrane, the gel and last three pieces of Whatman paper wetted with Cathode buffer. Put the top plate of the transfer unit, carefully. Run for 90 minutes at 2.5 mA/cm^2 membrane.

2.2.8.3. Blocking and Incubation With Antibodies

Non-specific binding sites were blocked by incubating the membrane in blocking solution for 2 h at room temperature or ON in the cold under shaking. The blocking was then followed by a washing step. Washing steps were performed by successive incubations of the membrane in an excess of TBS-T*:* one time for 15 minutes and three times for 5 minutes. Washing was performed at room temperature and under shaking.

The membrane was then placed into a metal box and incubated with the primary antibody for 1 h at room temperature under shaking. After removing the antibodies, the membrane was washed, incubated with the (1/10000) diluted secondary antibodies (Anti-mouse IgG, HRP linked with whole antibody (from sheep) and Anti-rabbit IgG, HRP linked with whole antibody (from donkey) (Amersham Life Science) or

Streptavidin conjugate HRP for 1 h at room temperature under shaking and washed again.

2.2.8.4. ECL-Detection

The solution (1,025 ml per mini blot) was freshly prepared (from ECL™ Plus detection system Amersham Biosciences, Freiburg), by mixing 1ml of solution A (0,4 mM p-coumaric acid (= 4-hydroxycinnamic acid), 100 mM Tris, pH 8,5) and 25 µl solution B (2,5 mM luminol (= 3-aminophthalhyrdrazide), 100 mM Tris, pH 8,5) and added to the protein side of the blot. After incubating for 3 min, excess detection reagent was drained off. The membrane was placed into a film cassette and exposed to a sheet of autoradiography film (BioMax X-rayfilm - Kodak) in a dark chamber. The expositions were performed from 10 sec to 1 h depending on the strength of the signal. The film was immediately developed.

Buffers:

Cathode buffer: 25 mM Tris/HCl pH 9.4; 40 mM Glycine; 10% Methanol.

Anode buffer 1: 0.3 M Tris/HCl pH 10.4; 10% Methanol.

Anode buffer 2: 25 mM Tris/HCl pH 10.4; 10% Methanol.

Blocking solution: 0.01% Tween-20 and 5% Skimmed milk in 1xTBS dissolved.

Wash buffer: 1 x TBS; 0.1 % Tween-20.

Stacking gel:

H_2O	13875 µl
30% Acrylamide:Bisacrylamide (29:1)	600 µl
0,5 M Tris/HCl pH 6,8	625 µl
10% SDS	25 µl
10% APS (Ammoniumpersulfate)	25 µl
TEMED	2,5 µl

Resolving gel:

	6%	8%	10%
H_2O	3.98 ml	3.48 ml	2.98 ml
30%Acrylamide:Bisacrylamide (29:1)	1.5 ml	2 ml	2,5 ml
1.5M Tris/HCl pH 8.8	1,86 ml	1,86 ml	1,86 ml
10% SDS	75 µl	75 µl	75 µl
10% APS	75 µl	75 µl	75 µl
TEMED	10 µl	10 µl	10 µl

2.2.8.5. Stripping of the Membrane

Keep the membrane wet if you plan to strip and re-use it. Once a membrane has dried, stripping will be ineffective. PVDF membranes may be stripped. Nitrocellulose generally does not strip well.

1. Wash membrane for one hour in **Stripping buffer** with gentle shaking at room temperature.
2. Rinse the membrane in blocking buffer, two times for 30 minutes.
3. Incubate the membrane in blocking solution for 1 h at room temperature. R
4. Repeat incubation with antibodies step.

Stripping buffer: 4 ml of 10 % SDS; 1.24 ml of 1M Tris/HCl pH 6,8; 140 µl of β-Mercaptoethanol and H_2O to 20 ml.

2.2.9. Immunostaining

Immunostaining cells on coverslips or minichambers:

1. Cells should be plated in 24 well Tissue Culture Plates (Greiner Labortechnik) containing coverslips (13 mm diameter) or Lab-Tek® Chambered Coverglass (Nalge Nunc International Corp., USA).

Fix cells when the cell density is not too high so the cells will not be crammed together. Usually plating at $5x10^4$ cells per well will be acceptable for next day use.

2. Prepare fixing solution just before use: I usually use 4% paraformaldehyde in phosphate-buffered saline PBS cells.
3. Remove media from wells and rinse cells once in ~1ml PBS (room temperature).
4. Remove PBS and add 0,5ml of 4% paraformaldehyde in PBS. Incubate at room temperature for 25 min.
5. Remove fixing solution and wash 2 times for 5min with PBS.

6. Permeabilize cells by incubation in 0.2 % Triton X-100 in PBS for 20 min at RT.

7. Block: Incubate fixed and permeabilized cells in blocking solution (3% BSA -bovine serum albumin in PBS) for 60 min.

8. Prepare primary antibody solutions diluted in blocking buffer (150 µl/coverslip).

9. Transfer coverslips to a box containing moist filter covered with parafilm. Mark a grid on the parafilm to help keep track of the coverslips. Lay the coverslips cell-side-up on the parafilm. Only transfer a few at time so they don't dry out.

10. Apply antibody solution to each coverslip. Incubate in primary antibody for 1 hour at RT or overnight at 4°C.

11. Wash the coverslips 3 times with PBS, 5 min/wash.

12. Prepare appropriate secondary fluorescent-labeled antibody solutions and apply to coverslips as above. Dilute antibodies in the blocking solution.

13. Incubate coverslips for 1 hour at RT in a dark cabinet.

14. Wash coverslips as above in PBS.

15. Mount the coverslips onto glass slides (can put two on each slide) using mounting media that contains an anti-bleaching agent (Vectashield Mounting Media with DAPI -4, 6-diamidino-2-phenylindole from Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010 U.S.A.).

Aspirate excess mounting liquid from the slide and seal coverslip in place with clear nail polish.

Fluorescence was analyzed using a Leica DMLB or Leitz Orthoplan microscope using a filter with maximum excitation at 480 nm and maximum emission at 520 nm for green fluorescence or one with maximum excitation at 550 nm and maximum emission at 600 nm for red fluorescence.

Confocal images were recorded using the Radiance 2000 confocal system produced by Bio-Rad Cell Science Division (Hemel Hempstead, United Kingdom) adapted to an Eclipse T300 inverted microscope (Nikon Corporation, Tokyo, Japan). The imaging objective was PlanApo, 60x (1.4 N.A., oil immersion, Nikon Corporation, Tokyo, Japan).

10% Paraformaldehyde (PFA**) stock (for preparation of 50 ml).** Weigh out 2g of paraformaldehyde (special EM grade). Add to 50 ml H2O in a 10ml backer glass and stir. The PFA will not go in to solution yet. Heat in 65°C water bath. Add 4-5 drops of 1M NaOH to the suspension and stir vigorously. The PFA will begin to dissolve. Continue heating until most of it is dissolved. Any remaining undissolved PFA can be filtered out using a 0.22 disposable filter.

3. Results

3.1. Modification of Sp3 Transcription Factor by SUMO

3.1.1. Overexpressed Sp3 is SUMOylated *in vivo*

At a functional level, there have been reports on the transcriptional properties of Sp3 in comparison with Sp1 based on co-transfection experiments into the insect cell line SL2. Sp1 acted as a strong transcriptional activator while Sp3 remained inactive or acted only as a very weak activator (Hagen et al., 1994; Majello et al., 1994; Dennig et al., 1995). The molecular basis for the inactivity of Sp3 has been mapped to an inhibitory domain located between the second glutamine-rich activation domain and the first zinc finger. Detailed mutational analyses further revealed that a single lysine residue (K551) is responsible for the low transcriptional activity of Sp3 (Fig 3.1). A database search highlighted that lysine 551 within the inhibitory domain falls into a SUMO consensus sequence, namely φ -Lys-X-Glu (where φ is a large hydrophobic amino acid, most commonly isoleucine or valine, and X is any residue), (Hay, 2001).

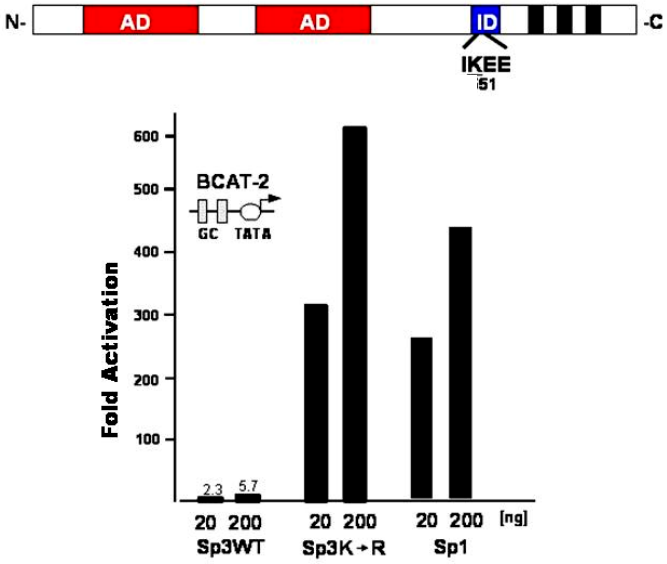


Fig. 3.1. Lysine 551 of Sp3 is responsible for the inactivity of Sp3. Color code: red - Activation Domains (AD); blue – Inhibitory Domain (ID); black – zinc finger DNA Binding Domain. (Diplomarbeit A. Ertmer).

3.1.1.1. Overexpression Systems

We asked whether Sp3 could act as a substrate for SUMO modification *in vivo*. The experimental approach used to address this question was to examine if overexpressed Sp3 in mammalian cells is subjected to SUMOylation. It was reported that SUMO-modified proteins have a ~20 kDa higher apparent molecular weight by Western blot analysis compared with unmodified proteins (Melchior, 2000).

In order to investigate SUMOylation of overexpressed Sp3, I first determined the best transfection system for mammalian cell lines. Ideally, an overexpression system would permit straightforward detection of Sp3 and potentially SUMO-modified Sp3.

Six different transfection techniques were tested (Fig.3.2), as well as three classical methods: the PEI, DEAE-dextran and Ca-Phosphate methods and three commercially available transfection reagents: Effectene (based on activated-dendrimer technology), FuGene6 (non liposomal transfection reagent) and Lipofectamine (liposomes based reagent). Ishikawa cells were transfected with an equal amount of pGL3-control vector used for general monitoring of transfection efficiency by the Luciferase Assay Method.

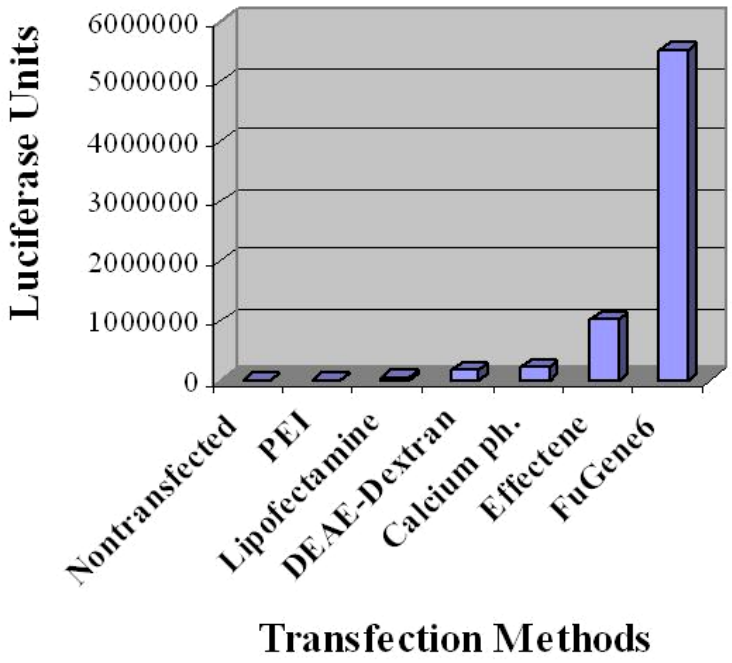


Fig. 3.2. Different transfection methods were tested for Ishikawa cell line.
One μg of pGL3-control vector was transfected using transfection methods as indicated. Luciferase activity was measured from samples containing the equal amount of protein. Control is Luciferase assay buffer and NT represents nontransfected cells.

Transfections were performed according to standard protocols and manufacturers instructions respectively (see Methods 2.2.4. Transient Transfection and Transfection Methods). As seen in Fig. 3.2, the best transfection was obtained with FuGene6 non-liposomal transfection reagent from Roche. During my experimental work I found this transfection method also reliable for other mammalian and *Drosophila* SL2 cell lines.

In attempting to discover a better transfection method, I tried out other transfection reagents like the commercial available JetPei, Lipofectamine Plus, Lipofectine or self prepared liposomes by combining different cholesterol derivatives, but ample augmentation in transfection efficiency was not observed. In conclusion, I decided to use the FuGene6 for most of the overexpression experiments.

3.1.1.2. Sp3 is Subject to Posttranslational Modification by SUMO1 and SUMO2 *in vivo*

Having established an appropriate expression system, we tried to answer the main question: whether transiently expressed Sp3 is SUMO modified *in vivo* by the endogenous SUMOylation machinery and if this modification is detectable by Western Blot analysis. Another key question was which member of the SUMO family is involved in Sp3 modification knowing that SUMO proteins from metazoa can be divided into two families, SUMO1 proteins and SUMO2/SUMO3 proteins.

A convenient solution chosen to address these questions applied the use of GFP fusion constructs that contained either wt Sp3 cDNA or Sp3 cDNA with a lysine to arginine amino acid exchange at the putative SUMO target site K551 (Sp3K/R). GFP fusion constructs for SUMO1 and SUMO2 were also used as they were expressed at a much higher level than the corresponding CMV-driven fusions with other tags (HA, FLAG), (data not shown). The GFP system has many powerful advantages. One can verify transfection efficiency by counting living cells expressing fluorescence and highly specific antibodies against GFP protein are commercially available.

I transfected gene constructs encoding GFP-Sp3WT or a mutant thereof (GFP-Sp3K/R) into Ishikawa cells alone (Fig. 3.3 lanes 1, 2, 3 and 6) or with SUMO1/2 GFP fusion constructs (Fig. 3.3 lanes 4, 5, 7 and 8). To avoid loss of

SUMOylation by highly active SUMO specific proteases, whole cell lysate extracts were prepared in denaturing SDS lysis buffer.

Only GFP-Sp3WT but not the GFP-Sp3K/R mutant was modified presumably by endogenous SUMO proteins, observed as a slower migrating Sp3 species with the wt protein (Fig. 3.3, lane 1, arrowhead). A slow-migrating form of GFP-Sp3WT was also detected in the presence of SUMO1 or SUMO2 fusion protein (Fig. 3.3, lanes 7 and 8, arrow). It was similarly abundant in the presence of SUMO1 or SUMO-2. Thus, both SUMO1 and SUMO2 were efficiently conjugated to Sp3 when overexpressed *in vivo*. In contrast, a slow migrating protein was absent with the GFP-Sp3K/R mutant although GFP-SUMO1 and GFP-SUMO2 were expressed at high levels (Fig. 3.3, lanes 4 and 5). The presence of two GFP-SUMO2 forms is most likely due to incomplete processing.

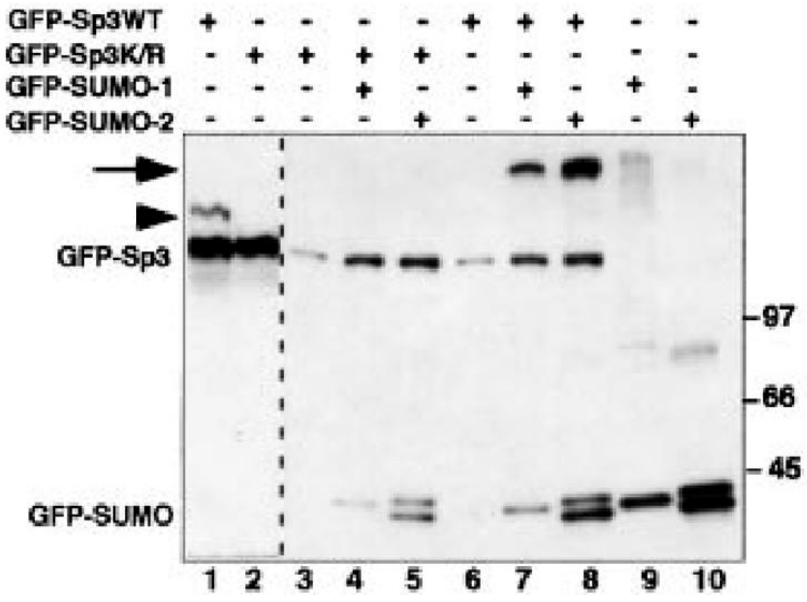


Fig. 3.3. Sp3 is SUMO modified *in vivo*.
GFP fusion vectors (3 µg) for GFP-Sp3WT, GFP-Sp3K/R, GFP-SUMO1 and GFP-SUMO2 were transiently transfected in Ishikawa cells as indicated. Cells were lysed and equal amounts of total cellular proteins (20 µg per lane) were separated by 6.0% SDS-polyacrylamide gel and blotted to PVDF membranes. Detection was by immunoblotting with anti-GFP antibodies. The arrowhead points to SUMO-modified GFP-Sp3WT, the arrow indicates GFP-SUMO-modified GFP-Sp3.

3.1.1.3. Posttranslational Modification of Sp3 by SUMO1 and SUMO2 is not Temporally Controlled

SUMO modification of proteins is a reversible process. SUMO moieties can be cleaved from their substrates by specific cysteine proteases like the yeast proteins Ulp1 (Li and Hochstrasser., 1999) and Ulp2 (Li and Hochstrasser., 2003) and their mammalian counterparts SUSP1 (Kim et al., 2000) and SENP1 (Gong et al., 2000). These enzymes also process the SUMO precursor.

The aim of this experiment was to determine how long after transfection of GFP-Sp3 and GFP-SUMO expression constructs, the GFP signal begins to be detectable in Western Blot. It was also designed to find out whether Sp3 SUMOylation after transient transfection is time dependent in the context of reversibility of this modification due to specific cysteine proteases.

Expression constructs for GFP-fused Sp3 were transient transfected into Ishikawa cell lines using FuGene6 transfection reagent, along with GFP-SUMO1 or GFP-SUMO2 respectively. Cell extracts were prepared at different time points in SDS lysis buffer with added protease inhibitors to inhibit SUMO-specific proteases (see Methods 2.2.7.1. Isolation of Proteins from Eukaryotic Cells).

The Sp3, SUMO1 and SUMO2 GFP fusion proteins were detectable after 12 hours post-transfection. Overexpressed Sp3 (GFP-Sp3 in Fig.3.4) was posttranslationally modified by endogenous SUMO (GFP-Sp3-SUMO) and GFP-SUMO1 or GFP-SUMO2 (Sp3-GFP–SUMO-GFP). A portion of transfected SUMO1 and SUMO2 was most likely not involved in the SUMOylation process (Free-SUMO-GFP in Fig.3.4). Another fraction of transfected GFP-SUMO1 or GFP-SUMO2 might be implicated in SUMOylation of others endogenous target proteins (* asterisk marked on the Fig.3.4).

Transiently transfected GFP-Sp3 and GFP-SUMO1 or GFP-SUMO2 started to be detectable by Western Blot analysis 12 hours post transfection. Once it was expressed, Sp3 was immediately modified by SUMO. The level of Sp3 SUMOylation appeared to be proportional to the level of total Sp3 protein therefore it was concluded that there

was not an optimum time point for maximum SUMOylation level. In conclusion, the Sp3-SUMO level seems to be constant suggesting a tight regulation of SUMOylation in mammalian cells.

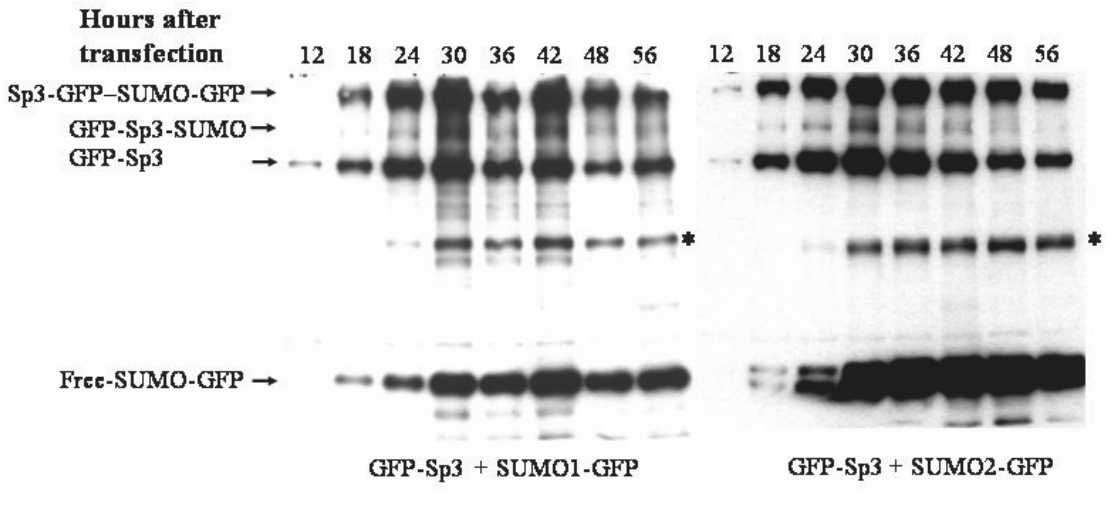


Fig. 3.4. Time course of Sp3 SUMOylation in vivo.
GFP fusion vectors for GFP-Sp3WT, GFP-SUMO1 and GFP-SUMO2 were transiently transfected in Ishikawa cells as indicated. Cells were lysed at different time points as indicated and equal amounts of SDS cell extracts (20 µg per lane) were separated on a 6.0% SDS-polyacrylamide gel and blotted to PVDF membranes. Detection was by immunoblotting with anti-GFP antibodies. The asterisk indicates one undetermined endogenous protein modified by SUMO-GFP.3.1.2. SUMOylation of Endogenous Sp3

3.1.2.1. Endogenous Sp3 Protein Pattern

At endogenous expression level, usually three Sp3 isoforms are detected, a 110-115 kDa Sp3 protein and two other approximately 60-70 kDa Sp3 species. It was shown recently, that *in vivo* four isoforms of Sp3 are expressed differing in the extent of their amino terminus part. Detailed mutational analyses suggest that all four isoforms are derived from alternative translational start sites. (Sapetschnig et al., 2004). My previous results validate that overexpressed Sp3 is SUMOylated *in vivo*.

Next we asked whether endogenous Sp3 is modified by SUMO.

To address this question, protein extracts were prepared from Ishikawa, HEK293

and mouse ES (Sp3-/-and WT) (Göllner et al., 2001) cells. Modified radioimmunoprecipitation (RIPA) buffer or SDS lysis buffer were used (see Methods chapter). To enhance Western Blotting signals, immunoprecipitations were performed using anti Sp3 specific antibody (Fig.3.5, lanes 5 and 6). Four distinct species, two slow migrating of more than 100 kDa and two fast migrating species of approximately 72 kDa were detected (Fig.3.5). Additional bands appeared, when cells were lysed directly in SDS containing buffer. Under these conditions seven to eight Sp3 species were visualized by immunoblots (Fig.3.5, lanes 3, 4 and 6). All signals were specific for Sp3 since extracts prepared from Sp3-deficient embryonic stem cells did not exhibit any background (Fig.3.5, lane 2). Sp1 transcription factor was also visualized and shown to be expressed as no modified and phosphorylated forms (Fig.3.5, lane 1).

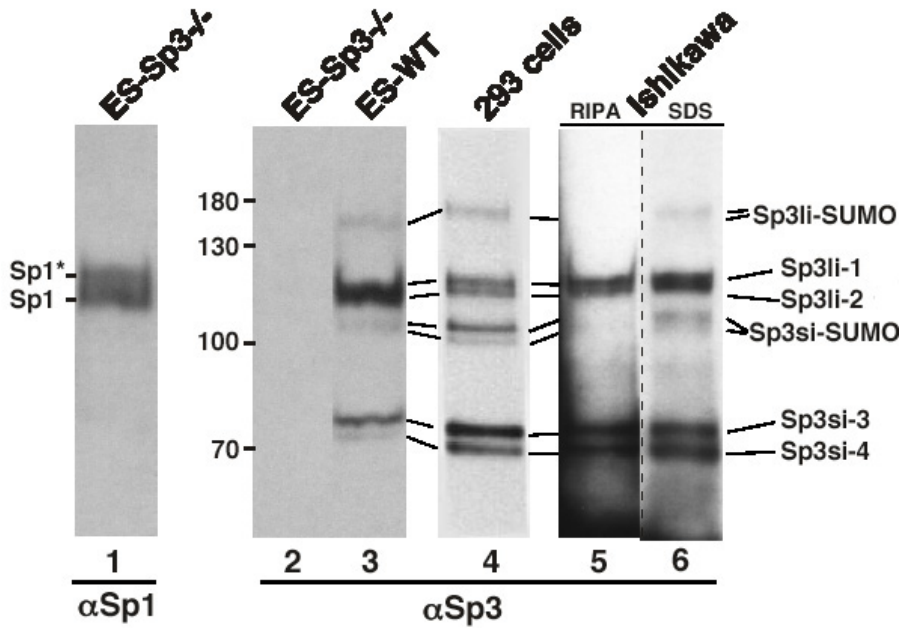


FIG 3.5. Complexity of endogenous Sp3 protein expression. Western blot analyses of Sp1 and Sp3 in wild-type ES (ES-WT, lane 3), Sp3-deficient ES (ES-Sp3-/-, lane 1 and 2), 293 (lane 4) and Ishikawa cells (lanes 5 and 6). Cells were lysed with SDS-containing buffer (lanes 1-4 and 6) or with RIPA buffer (lane 5) and proteins were separated through 6% SDS polyacrylamide gels. After blotting, PVDF membranes were incubated with Sp1- or Sp3-specific antibodies as indicated. Abbreviations are: Sp3li-1 and 2, long isoforms of Sp3; Sp3si-3 and 4, small isoforms of Sp3; Sp3li-SUMO and Sp3si-SUMO, SUMO-modified long and small isoforms of Sp3. For Sp1, the asterisk indicates the phosphorylated form.

High resolution immunoblot analyses with antibodies directed to the C-terminus of Sp3 resulted in a striking complex protein pattern that allowed detection of four different species, contrary to previously published results showing only one long Sp3 isoform (Suske, 1999). It should be noted that SUMO-modified isoforms were observed in all cases where SDS extracts were prepared for Sp3 expression analyses. In those cases where nuclear extracts were generated in the absence of protease inhibitors, these posttranslational modified proteins were not observed presumably since hydrolases cleaved the Sp3-SUMO isopeptide bond.

3.1.2.2. Sp3 in Sp1-Deficient Mouse ES Cells

The structural similarity between Sp1 and Sp3 as well as their ubiquitous expression suggested originally that they have similar properties and exert similar functions. However, biochemical and biological studies highlighted significant differences, including different knockout phenotypes (Bouwman et al., 2000; Göllner et al., 2001), different posttranslational modifications and expression of different isoforms (Braun et al., 2001; Sapetschnig et al., 2002; Sapetschnig et al., 2004).

The following experiments determine whether Sp3-SUMOylation or the Sp3 expression pattern are affected under Sp1 knockout conditions.

Mouse ES cells derived from Sp1-/-, Sp1+/- and wt embryos were maintained in culture in the presence of LIF to prevent differentiation. SDS total cell extracts were prepared in all cases to preserve SUMO modification (see Methods).

There was no obvious difference in SUMOylation of Sp3 long isoforms (liSp3-SUMO) under Sp1 KO conditions as shown in Fig.3.6.A. Interestingly, the expression level of the small Sp3 isoforms decreased in Sp1 deficient ES cells while expression of the long isoforms remained constant.

As a control, the membrane was probed with anti Sp1 serum (Fig.3.6.B). The expression pattern of Sp1 was seen depicted as follows: in WT ES cells (Sp1+/+) the Sp1 protein was detected along with its phosphorylated form (Sp1-P), in heterozygous cells (Sp1+/-) the expression of both Sp1 and Sp1-P was reduced and in Sp1 knockout

cells (Sp1-/-) only the N-terminus of Sp1 was expressed, resulting in a faster migrating Sp1 form (Marin et al., 1997). It should be noted that in Sp1 KO, only the Sp1 DNA Binding Domain was deleted.

In conclusion, Sp3-SUMO modification in ES cells appears not to be altered. A significant shift towards the long isoforms of Sp3, however, was observed in Sp1-/- ES cells demonstrating that Sp3 isoform expression can change *in vivo*. In addition, this observation suggests that the long isoforms of Sp3 may take over Sp1 functions under Sp1 knockout conditions.

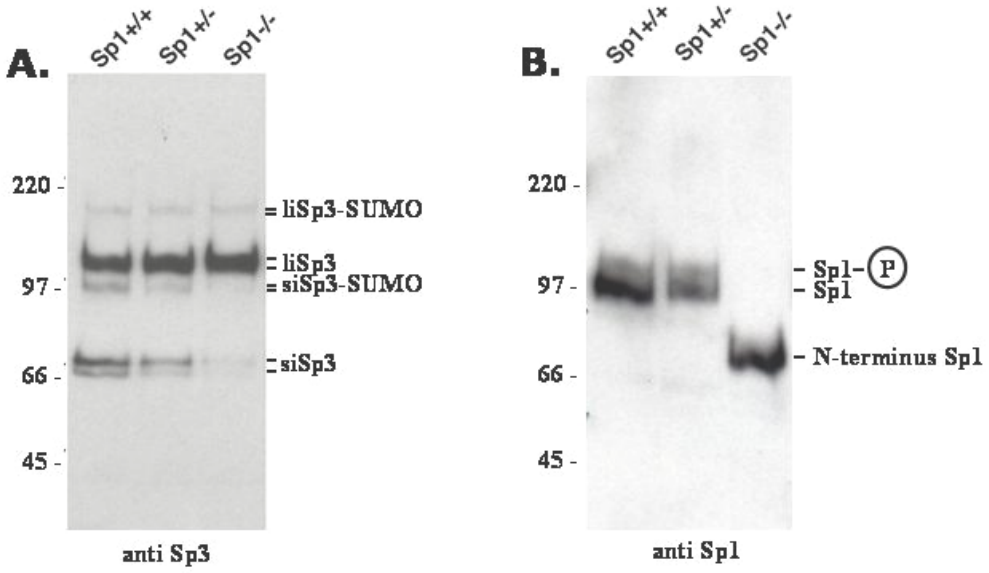


Fig.3.6. Sp3 and Sp1 expression in Sp1-deficient mouse ES cells.
A. Mouse ES cells derived from embryos as indicated above were lysed with SDS containing buffer and Sp3 expression was analyzed by Western blotting using anti-Sp3 antibody (SC).
B. The same blot was probed with anti-Sp1serum.
Abbreviations are: Sp3li-1 and 2, long isoforms of Sp3; Sp3si-3 and 4, small isoforms of Sp3; Sp3li-SUMO and Sp3si-SUMO, SUMO-modified long and small isoforms of Sp3. For Sp1, the Sp1-P indicates the phosphorylated form.

3.1.3. The Full-Length Overexpressed Sp3 SUMOylation

3.1.3.1. *In vivo* Reconstitution of Sp3 Isoforms

The experiments in all previously published studies about Sp3 (more than 500 citations in Medline) were performed with N-terminally truncated versions of Sp3. Recently however, human genomic DNA sequences were identified that encompass three exons coding for additional 85 N-terminal amino acids of Sp3 (Oleksiak et al, 2002). Within these 85 aa, two AUG codons are present (Sapetschnig et al., 2004).

The full-length Sp3 open reading frame that starts with a classical AUG initiation codon was cloned in our lab.

We asked then whether expression of the four Sp3 isoforms that were detected at endogenous expression levels (see chapter 1.2.1) could be reconstituted *in vivo* after transfection of appropriate expression constructs.

Full-length Sp3 cDNA and lysine 551 mutant cDNA were cloned into the CMV promoter driven expression vector pN3 derived from pEGFP-N3 (Clontech), in which the EGFP tag was removed. An Sp3 sequence lacking the codons for the first 13aa (Δ13 Sp3) and its lysine mutant (Δ13 Sp3 551K/R) were used. For cloning strategy see Methods chapter.

After transfection of the wild-type full length (FL) Sp3 cDNA, two long isoforms of Sp3 were expressed in HEK293 cells at the similar level (Fig.3.7. A-1). In contrast, after expression of Δ13 Sp3 (encoding 769 aa from the 781 aa in WT), only one of Sp3 long isoforms was detectable (Fig.3.7. A-2).

The endogenous Sp3 signal by Western Blot was not as strong as the signal of overexpressed Sp3 (Fig.3.7 compare line 2 with lines 3-6). For a better comparison of endogenous Sp3 expression pattern and Sp3 overexpressed in HEK293 cells, a longer exposure of lane 2 is shown in line1- marked Exp in the figure. The two small isoforms of Sp3 were produced at low level after overexpression of Sp3 constructs in HEK293 cells (Fig.3.7. B lines 3-6). Another important observation that results from this experiment is that transfected Sp3 (both full length and Δ13 Sp3) were SUMO modified *in vivo* (lines 3 and 5) but the 551 lysine mutants (lines 4 and 6) were not.

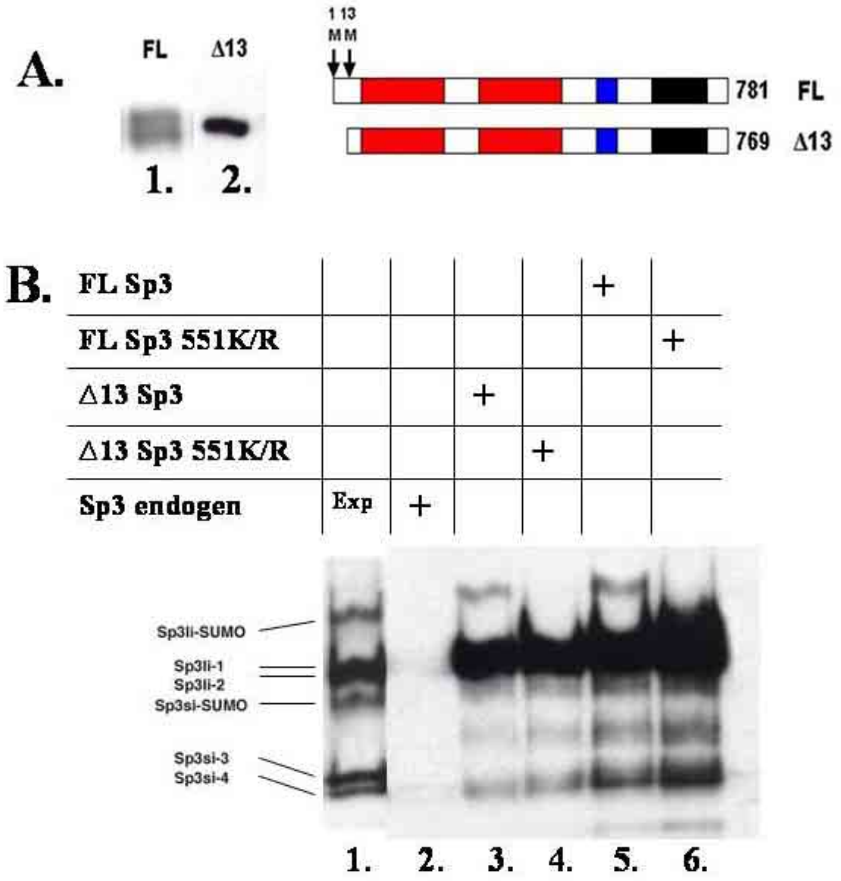


Fig.3.7. In vivo SUMOylation of transient expressed Sp3.

A. Separation of long Sp3 isoforms in long running SDS polyacrylamide gel after transfection of pN3-Sp3 full length (FL) and pN3-Sp3 Δ13 expressions constructs.

Schematic drawing: Depicted protein structure of Sp3 full length (FL) and Sp3 protein lacking the N- terminus 13 aa (Δ13). Arrows indicate the methionine residues 1 and 13 translated from the in frame start codons. Color code: red - Activation Domains; blue – Inhibitory Domain; black – zinc finger DNA Binding Domain.

B. Transfection of expression constructs encoding full length Sp3 cDNA and Sp3 cDNA lacking the first 13 aa (pN3-Sp3 FL, pN3-Sp3 551K/R-FL and pN3-Sp3 Δ13, pN3-Sp3-551K/R Δ13 as indicated).

Total cell SDS extracts were prepared and proteins separated through 6% SDS polyacrylamide gels. After blotting, PVDF membranes were incubated with Sp3 specific antibody.

Abbreviation: Exp- longer exposure time of control line (**2**.) in order to detect endogenous Sp3 signal. For detection, the PVDF membrane was film exposed for additional time.

Sp3li-1 and 2, long isoforms of Sp3; Sp3si-3 and 4, small isoforms of Sp3; Sp3li-SUMO and Sp3si-SUMO, SUMO-modified long and small isoforms of Sp3.

Expression of the four Sp3 isoforms was reconstituted *in vivo* after transfection of appropriate expression constructs but the small isoforms are produced at very low level. The same phenomenon was observed after Sp3 expression in insect Schneider cells (SL2 cells) (see Fig. 3.24 D).

There is only a tiny difference (13 aa) between Sp3 long isoforms (at approx. 115 kDa) (Fig.3.7. A- schematic drawing), making discrimination among them by Western Blot very difficult. Despite that, one can observe the separation of isoforms after transfection of full length Sp3 expression construct, but not of a Δ13 Sp3 by Western Blot analysis.

After overexpression, Sp3 was SUMO modified at lysine 551 *in vivo* in both, the Sp3 full length and the Δ13 Sp3 proteins.

3.1.3.2. Additional Potential SUMO Sites

My previous results verify that overexpressed Sp3 is SUMO-modified at lysine 551 *in vivo*. Two additional potential SUMOylation sites are present at lysine 9 and 120 (Fig. 3.9). Lysine 9 is located within the first 13 aa of the Sp3 sequence, which means that the consensus site is present only in the longest isoform. Located in the first glutamine-rich activation domain, the second putative SUMOylation site, K120 exists in both long isoforms.

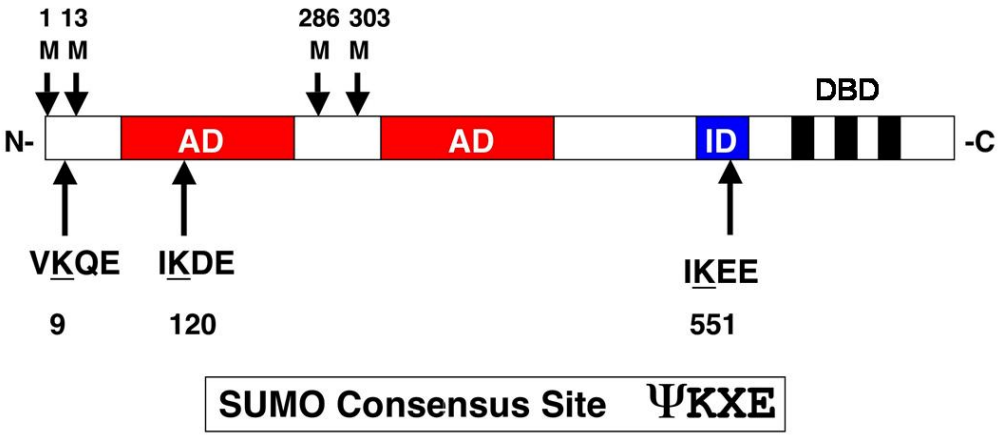


Fig.3.9 Other potential SUMOylation sites in Sp3. The length of Sp3 refers to the full-length isoform (781 aa). Red boxes indicate activation domains (AD) that are rich in glutamine residues. The black boxes represent the zinc fingers DNA binding domain (DBD). ID in the blue box indicates the inhibitory domain.

The next experiments were designed to determine if lysine 9 or/and 120 are targets for SUMO modification *in vivo.*

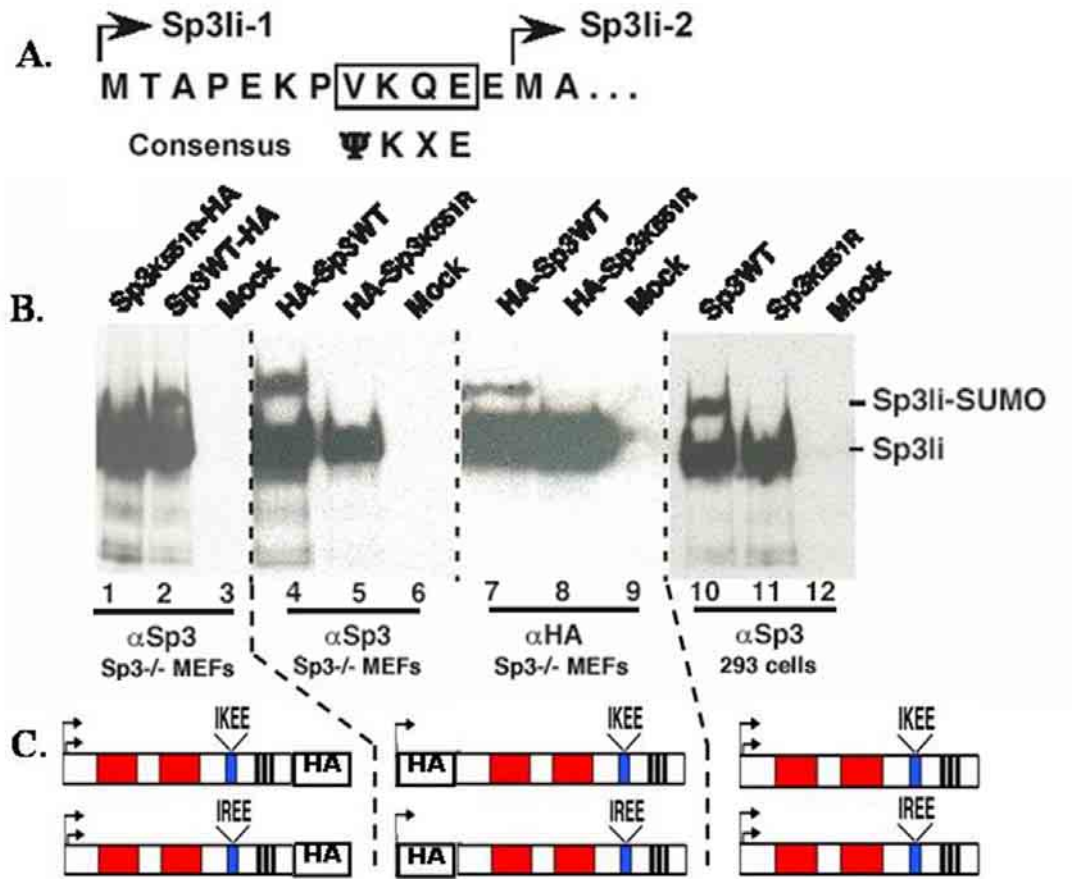


FIG. 3.10. Posttranslational modification of full-length Sp3 by SUMO.
A. N-terminal amino acid sequence of the Sp3 protein. The potential SUMO target sequence VKQE present in the longest Sp3 isoform (Sp3li-1) is boxed. The SUMO target consensus sequence reads ΨKXE.
B. *In vivo,* Sp3 becomes modified by SUMO exclusively at lysine K551. CMV-driven expression plasmids for N-terminally HA-tagged (HA-Sp3WT and HASp3K551R), C terminal HA-tagged (Sp3WT-HA and Sp3K551R-HA) or untagged Sp3 (Sp3WT and Sp3K551R) as indicated were transfected into Sp3-/- MEFs (lanes 1 to 9) or 293 cells (lanes 10 to 12). Cells were lysed with SDS-containing buffer and Sp3 expression was analyzed by Western blotting using anti-Sp3 or anti-HA antibodies as indicated.
C. Schematic drawings of tagged and untagged Sp3. Color code: red - Activation Domains; blue – Inhibitory Domain; black – zinc finger DNA Binding Domain.

To address this question, I cloned full-length versions of wild-type Sp3 or the K551R mutant in expression vectors such that Sp3 was either HA-epitope tagged at the N-terminus or the C-terminus, respectively as seen in Fig.3.10-C.

The expression constructs were transfected into Sp3-/- MEFs and HEK293 cells. Cell extracts were prepared in SDS lysis buffer and subjected to immunoblot analyses.

SUMO-modified Sp3, visualized as a slower migrating species, was exclusively detected with the wild-type Sp3 constructs, but not with the Sp3K551R constructs (Fig. 3.10). If one of lysine 9 or 120 were SUMO modified, a slower migrating band would have been observed in all Sp3K551R Western Blot lanes in the same range as SUMO-Sp3 in Sp3 WT.

This result strongly suggests that only K551 becomes SUMO-modified and not K9 and K120 or any other lysine residue of the Sp3 sequence. In other proteins containing multiple SUMO sites one can observe by Western Blot analysis the non-modified protein, and a ladder of slower migrating bands that correspond to the number of lysines SUMOylated (for example as seen in SUMOylation of Topoisomerase I , Mao et al., 2000).

In Sp3 WT proteins, additional slower migrating bands were not present except the band shown to represent SUMO modification of Sp3 at Lysine 551.

3.2. Subcellular Localization of Sp3

3.2.1. Optimization of the Immunofluorescence Protocol

The nucleus was one of the first intracellular structures to be identified by microscopy, but its functional organization is still poorly understood. It has become apparent that the nucleus is highly compartmentalized but extremely dynamic. Nuclear factors can display a diffuse nuclear distribution or are localized in distinct structures, such as speckles, paraspeckles, nucleoli, Cajal bodies, gems and Promyelocytic Leukemia bodies, that show a punctuate staining pattern when analyzed by indirect immunofluorescence microscopy (Spector et., 2003).

The following analysis addresses the subcellular localization of the transcription factor, Sp3. Initially, visualization of Sp3 was optimized. Different protocols for immumofluorescence and different antibodies were tested in order to determine the

most suitable. A detailed immunostaining protocol is described in the Methods chapter. Before bringing concrete evidences about Sp3 localization, I want to point out some of the problems, which appeared during distinct immunofluorescence protocol steps. In principle, I used insect SL2 or different mammalian cell lines plated on round coverslips or on Permanox 8 well immunostaining chambers. Fixation of cells was performed with freshly prepared 4% paraformaldehyde in phosphate-buffered saline.

I found permeabilization one of the most critical steps in immunostaining. Any alteration in incubation time or permeabilization buffer preparation led to artifacts, as demonstrated in Fig.3.11. The permeabilization buffer always contained a strong detergent (Triton X, NP-40) able to permeabilize the cytoplasmic membrane and the nuclear envelope, in order to allow antibodies and other reagents to reach cellular compartments.

In Fig. 3.11.A, following permeabilization using TritonX the Sp3 signal is detectable in the nucleus. However, when a mild detergent (Saponin), able to permeabilize cytoplasmic membrane but not the nuclear envelope was used, the Sp3 signal was detected in the cytoplasm and around nuclear periphery (Fig. 3.11.B). Likely, this localization reflects an artifact.

Different localizations of Sp3 were also observed when the sample incubation with permeabilization buffer was less then 10 min.

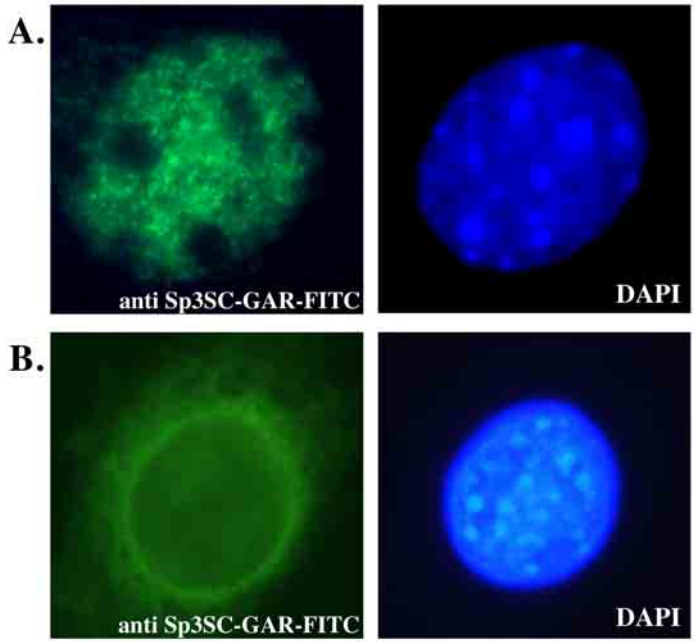


Fig. 3.11. Sp3 localization under different permeabilization conditions.
A. Nuclear subcellular localization of Sp3 under Triton X permeabilization conditions for 20 min.
B. Sp3 signal at nuclear periphery after replacing 0.02 % Triton X with 0.05 % Saponin at membranes permeabilization immunodetection protocol step.
Sp3 was detected by immunostaining in MEF cells with a rabbit anti-Sp3 antibody and a FITC conjugated secondary antibody. Control staining of nucleus was performed with DAPI.

Another significant step in establishing the immunostaining technique was testing the primary antibodies at different incubation concentrations in order to diminish background fluorescence.

Fig.3.12 compares cytoplasmic background caused by anti Sp3 serum with a commercial anti Sp3 antibody purchased from Santa Cruz.

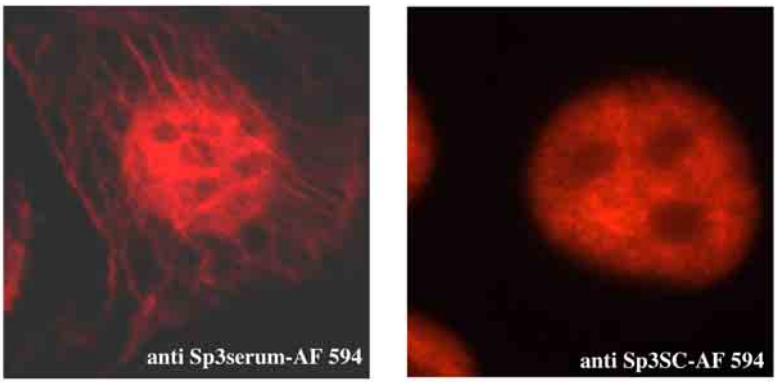


Fig 3.12. Comparison of Sp3 localization in Ishikawa cells by using two different primary antibodies for immunodetection.
Anti Sp3 serum or anti Sp3 commercial available antibody (from Santa Cruz) and AF594 conjugated secondary antibody were used for endogenous Sp3 detection by immunostaining.

The final protocol step involves incubation with fluorophores conjugated to a secondary antibody. When two different sets of antibodies are used to detect simultaneously two different proteins, it is recommended that fluorophores with disparate spectra are used. The fluorophores are visualized using fluorescence microscopy, and independently distinguished using filter sets specific for each color. Images are captured with a charge-coupled device (CCD) camera.

An observation was that fluorescence emitted by red fluorophores (AF 594, Cy5, Texas RED) transmits more clearly through cells than blue (AF350) or green (FITC), because cells absorb less energy at longer wavelengths.

At last, coverslips were mounted onto glass slides using Vectashield mounting media with DAPI and sealed with nail polish.

3.2.2. Endogenous Sp3 Localizes to Nucleus

Having established an optimized immunostaining technique, I tried to determine Sp3 subcellular localization.

The nuclear distribution of Sp3 was carefully examined and compared to the localization pattern of other Sp family members, In addition, the effect of overexpression on Sp3 localization was determined.

3.2.2.1. Sp3 is Distributed Throughout Nucleus

I noticed from the preliminary Sp3 immunofluorescence experiments that Sp3 is predominantly located within the nucleus. Subsequent analysis investigated whether Sp3 displays a particular pattern or is associated with specific structures.

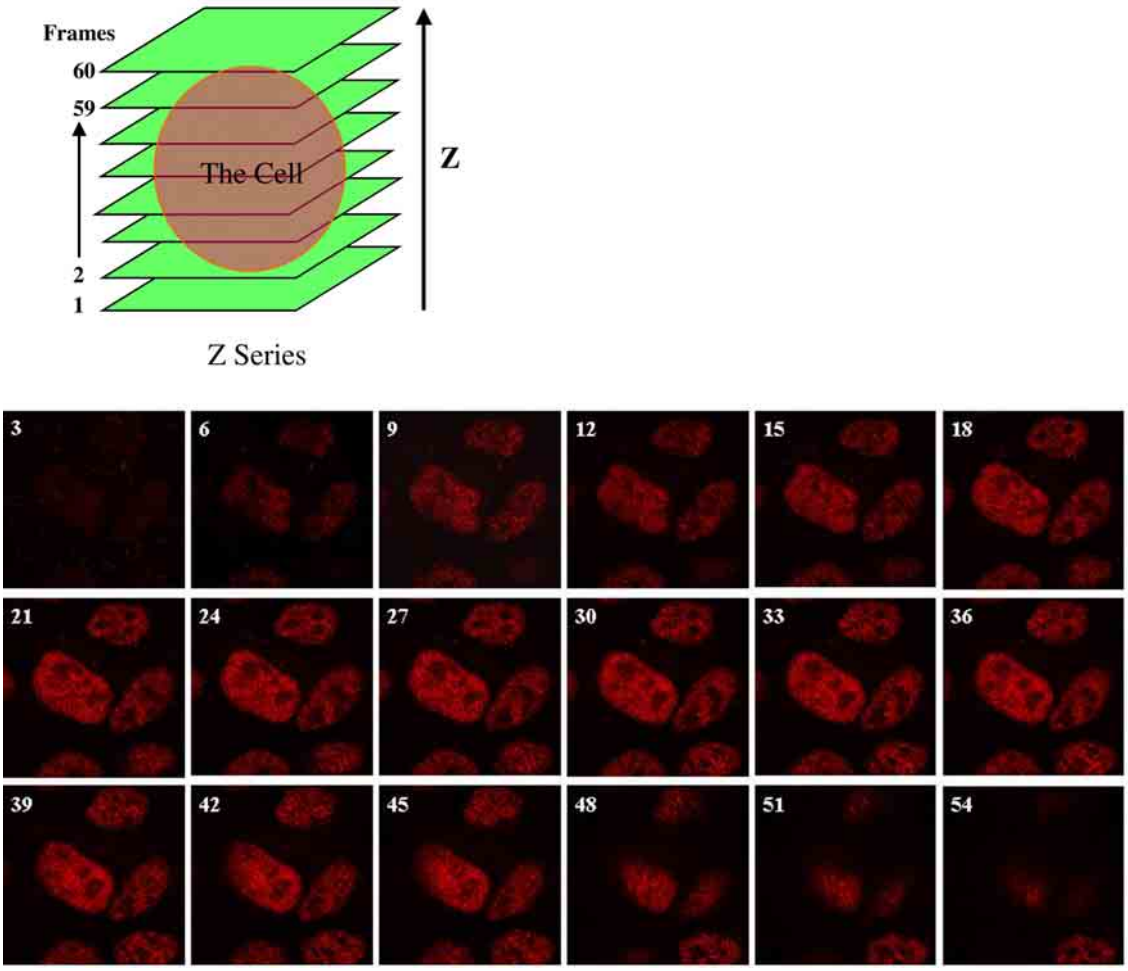


Fig. 3.13. Endogenous Sp3 is present throughout the nucleus in Ishikawa cells.
In the upper scheme the principle of this microscopy method is depicted. The cell is divided in several confocal planes throughout the Z axis and the images of each plane are recorded separately.
Endogenous Sp3 localization was detected with a rabbit anti-Sp3 antibody (SC) and a goat anti rabbit – Alexa Fluor 594 conjugated secondary antibody. The figure shows the image of every third confocal plane.
Confocal Z-stacks were recorded using the Radiance 2000 confocal system produced by Bio-Rad Cell Science Division (Hemel Hempstead, United Kingdom) adapted to an Eclipse T300 inverted microscope (Nikon Corporation, Tokyo, Japan).

Ishikawa cells were prepared for immunostaining according to the protocol (see Methods). Detection of endogenous Sp3 was performed using a rabbit anti-Sp3 antibody and a goat anti rabbit – Alexa Fluor 594 (GAR-AF-594) conjugated secondary antibody. Permanent slides were used for Z series recordings with the Bio-Rad confocal system specially designed to record "slices" through the cell, as seen in the upper schematic drawing from Fig 3.13. The system was set to record 0.5 μm stacks resulting in approximately 60 pictures from one cell.

Fig.3.13 shows endogenous Sp3 localization in the nucleus of Ishikawa cells by displaying every third frame of the Z series. This experiment demonstrates that Sp3 is distributed throughout nucleus and this distribution is not uniform, but rather with random holes giving the appearance of a "sponge-like structure".

3.2.2.2. Comparison with Localization Pattern of other Sp Family Members

The structural similarity of the four Sp family members suggests that they are evolutionally closely related, although biochemical and biological studies have highlighted significant differences between them (Suske, 1999). To resolve the controversy surrounding differences or similarities between subcellular localization patterns of Sp family members, subcellular localization patterns were analyzed by immunofluorescence assays.

Endogenous Sp1 and Sp2 were detected in HEK293 cells, using rabbit anti Sp1 and rabbit anti Sp2 sera, respectively. The Sp4 transcription factor could not be detected, neither with a rabbit serum produced and purified in our lab nor with a commercial available antibody from Santa Cruz. One possible explanation could be that the expression level of Sp4 in HEK293 cell line is very low.

Goat anti rabbit secondary antibodies conjugated with two fluorophores: FITC (green) and Alexa Fluor 594 (red) were used.

Sp1 and Sp2 transcription factors were located predominantly in the nucleus and the subcellular localization patterns are similar to Sp3 (Fig.3.14).

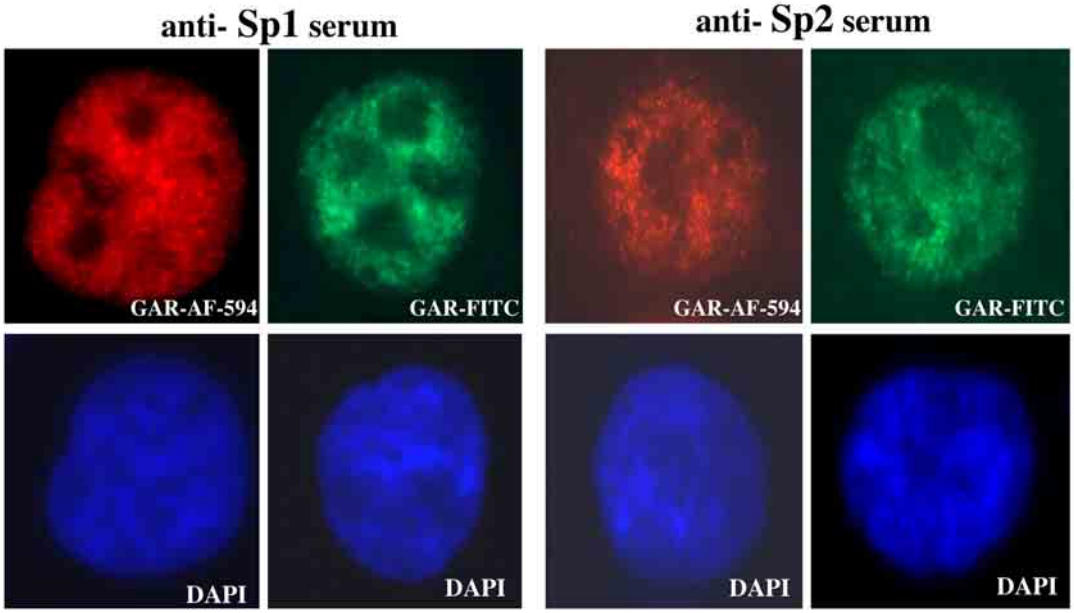


Fig 3.14. Subcellular localization of the Sp family members Sp1 and Sp2 in HEK293 cells. Sp1 and Sp2 were detected by immunostaining with a rabbit anti-Sp1 or an anti-Sp2 serum as indicated and an AF594 or FITC conjugated secondary antibody. Control staining of nuclei was performed with DAPI.

3.2.2.3. Overexpressed Sp3 Localization Pattern

Next, whether overexpressed Sp3 protein displayed the same localization pattern as endogenous Sp3 was established.

Thus, I cloned the full-length Sp3 cDNA into the MCS of pEGFP-C1 (Clontech), which has been optimized for brighter fluorescence. The enhanced GFP variant, EGFP, is up to 30 times brighter than wild-type GFP and is more stable. Sp3 full-length was expressed as a fusion protein with EGFP (as seen in Fig. 3.15 schematic drawing). The expression construct was transiently transfected into Ishikawa cells.

Visualization of Sp3 was achieved by the intrinsic green fluorescence of the EGFP moiety. Transiently transfected Sp3 expressed as a fusion protein with EGFP exhibited a similar localization pattern as endogenous Sp3 protein (Fig. 3.15).

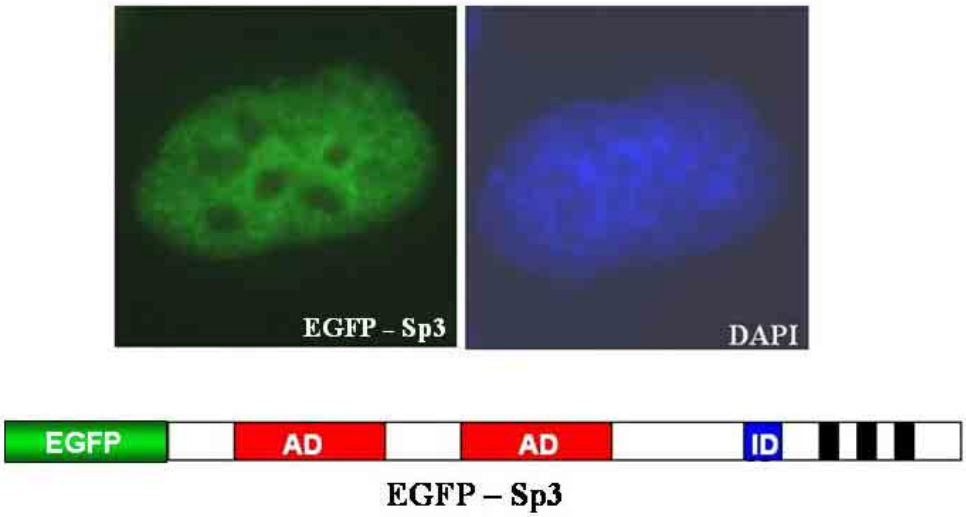


Fig **3.15. Expression pattern of overexpressed EGFP - Sp3 fusion protein.**
The EGFP -Sp3 expression construct was transiently transfected into Ishikawa cells. Visualization of Sp3 was by the intrinsic green fluorescence of the GFP moiety. Control staining of nuclei was performed with DAPI.

Conclusions:

1. The Sp3 transcription factor was predominantly located within the nucleus.

2. Visualization of endogenous Sp3 by immunofluorescence showed a sponge-like, diffuse appearance.

3. Evolutionally closely related Sp family members Sp1 and Sp2 were also located within the nucleus and their subcellular localization patterns were similar to Sp3.

4. Overexpressed Sp3 displayed a similar localization pattern as endogenous Sp3 protein.

3.2.2.4. Subcellular Localization of Sp3 Isoforms and SUMOylation – Deficient Mutants

Western blot analysis revealed that four different isoforms of Sp3 are expressed (see Results Chapter 3.2.1). The most obvious difference between the two long isoforms and the two small isoforms of Sp3 is the presence of either two or just one single glutamine-rich domain. Both Q-rich domains can act as strong activation

domains on their own when fused to a heterologous DNA-binding domain. The transcriptional activity of the two long isoforms strongly depends on the promoter settings whereas the small isoforms appear to be inactive (Sapetschnig et al., 2004).

I wanted to know whether the apparent differences in regulatory properties of the small and long isoforms of Sp3 reflected differences in their subcellular localization. To achieve this, I examined the localization of Sp3 isoforms in MEF and *Drosophila* SL2 cells.

Wild-type mouse embryonic fibroblasts (MEF) cells were used to investigate the localization of endogenous Sp3 and Sp3-deficient MEFs (MEF KO nr.6- PhD Thesis H. Göllner) were included as a negative control. To analyze localization of Sp3 long isoforms, I used Sp3 KO MEFs stable transfected with Sp3 FL and the SUMOylation-deficient mutant Sp3 K551R FL (Diplomarbeit T. Mennenga). To investigate the short isoforms, MEFs KO were stably transfected with constructs expressing short isoforms and short isoforms with a small deletion (SD) within the inhibitory domain (containing IKEE SUMO motif), (MEF- Ubi-Sp3-W, and MEF-Ubi-Sp3-WSD described in PhD Thesis H. Göllner).

Drosophila appears to have only four SP transcription factors, as opposed to nine in mammals (Suske et al., 2005). The reason for using *Drosophila* - derived SL2 cells was the absence of endogenous Sp3 expression. SL2 cells were transfected with the appropriate expression constructs for long (pPac-Sp3 FL new) and small wild-type Sp3 isoforms (pPAC-Sp3), as well as with the corresponding SUMOylation -deficient mutants (pPac-Sp3 K/R FL new and pPAC-Sp3 K/D), schematically represented in Fig 3.16.

Detection of Sp3 protein in both cell types was achieved by immunostaining with a rabbit anti-Sp3 antibody and a FITC- (Fig. 3.16 A) or AF594 conjugated (Fig. 3.16 B) secondary antibody.

Immunostaining with anti-Sp3 antibodies revealed that MEFs and SL2 cells types, the wild-type isoforms and the SUMOylation-deficient mutants were located in the nucleus and exhibited a sponge-like, diffuse appearance.

These results show that the differences in the activation capacity of the various

isoforms and mutants are not due to differences in their subcellular or subnuclear localization.

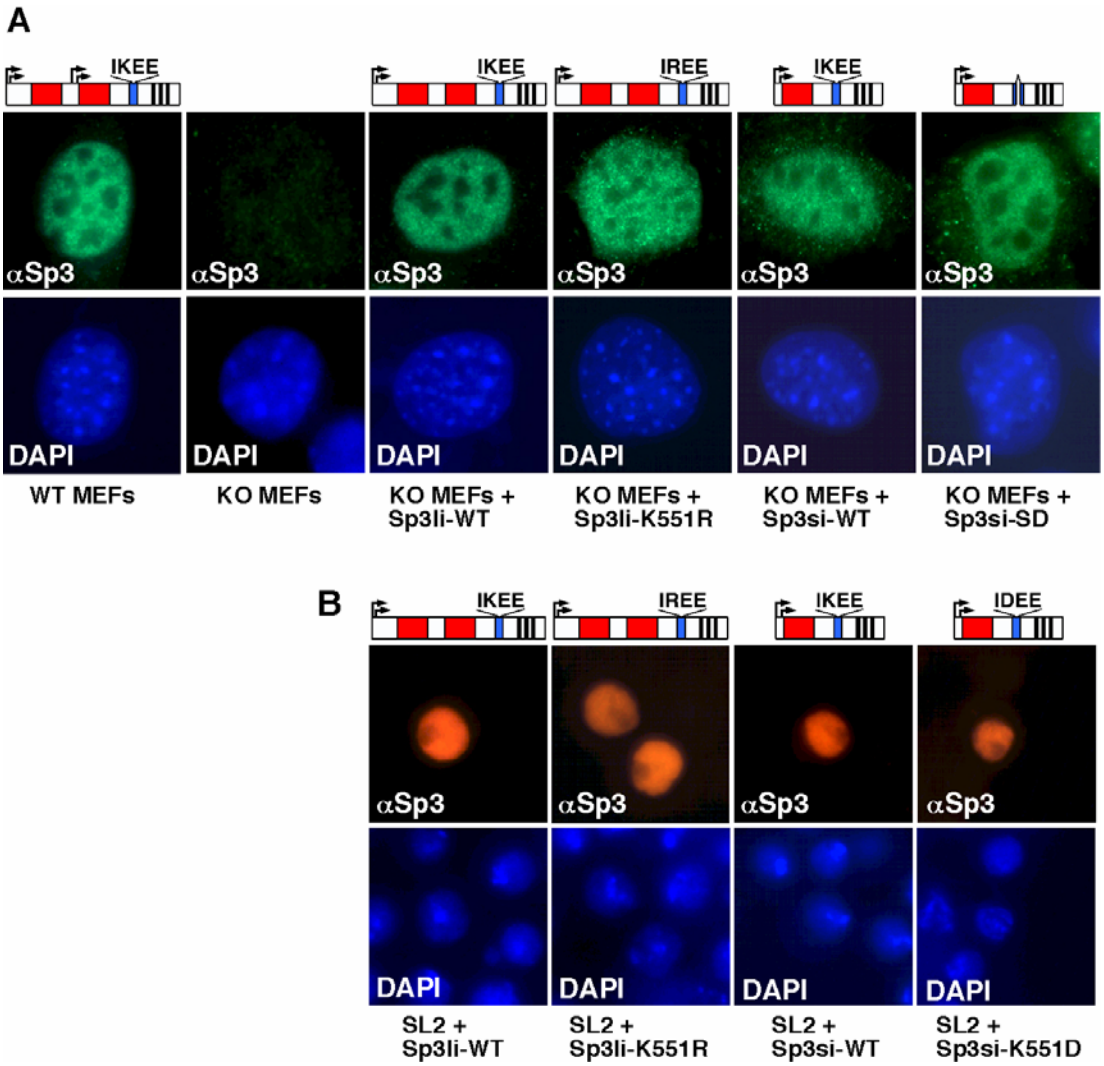


FIG. 3.16. Subcellular localization of Sp3 isoforms and corresponding SUMOylation-deficient mutants in MEFs and SL2 cells.

A. Sp3-/- MEFs stably transfected with expression constructs for the long or the small isoforms (Sp3li-WT and Sp3si-WT, respectively) and corresponding SUMOylation-deficient mutants (Sp3li long isoform -K551R and Sp3si small isoform-SD) are shown. The Sp3siSD mutants contain a thirteen amino acid deletion including the IKEE SUMOylation motif. WT MEFs and mock-transfected Sp3-deficient KO MEFs were included in the analyses.

B. SL2 cells were transiently transfected with one µg of expression constructs for Sp3 isoforms and mutants as indicated.

Sp3 was detected by immunostaining with a rabbit anti-Sp3 antibody and a FITC- (A) or AF594 conjugated (B) secondary antibody. Control staining of nuclei was performed with DAPI.

3.2.3. The Subcellular Localization of Sp3 is NOT Altered Upon SUMO Modification

The cell nucleus is highly compartmentalized and extremely dynamic. Many nuclear factors, in addition to being diffusely distributed throughout nucleoplasm, accumulate in specific and distinct nuclear subcompartments, such as nucleoli, speckles, Cajal bodies and promyelocitic leukemia (PML) bodies (Seeler and Dejean, 2003). Posttranslational modification by SUMO has been shown to regulate subcellular localization of many targets including RanGAP, the IKB kinase regulator NEMO, the tumor suppressor PML and many other SUMO-modified proteins including transcription factors, chromatin modifiers, and proteins involved in genomic maintenance (Zhong et al. 2000).

It was verified in the previous chapters (1.1, 1.2) that Sp3 is a SUMO target in vivo. The following experiments determine if Sp3 subcellular localization pattern is affected by SUMO. Two main questions are addressed: firstly do endogenous Sp3 and SUMO colocalize? Secondly what is the effect of SUMO overexpression on Sp3 nuclear distribution?

3.2.3.1. Subcellular Localization of Endogenous SUMO1

To seek out the answer of the first question, several double immunostaining experiments were performed to detect subcellular localization of endogenous Sp3 and SUMO1 simultaneously. Ishikawa and MEF cells were used for this experiment.

Fig. 3.17.A shows the subcellular distribution of endogenous Sp3, detected with rabbit anti-Sp3 antibody and Goat Anti Rabbit (GAR) conjugated with FITC, and endogenous SUMO1, visualized using a monoclonal anti SUMO1 and goat anti mouse (GAM)-Texas Red conjugated secondary antibody.

Fig. 3.17.B details SUMO1 subcellular localization in Ishikawa cells, detected with monoclonal anti SUMO1 and GAM-Texas Red and in MEFs cells, detected with rabbit anti-SUMO1 (Santa Cruz) antibody and a Cy3 conjugated secondary antibody.

Endogenous SUMO1 exhibits a different nuclear pattern than that of the Sp3 transcription factor and appears to not influence its localization. SUMO1 displayed a

diffuse nuclear distribution but in addition appeared do be accumulated at the rim of the nucleus and in a few nuclear dots.

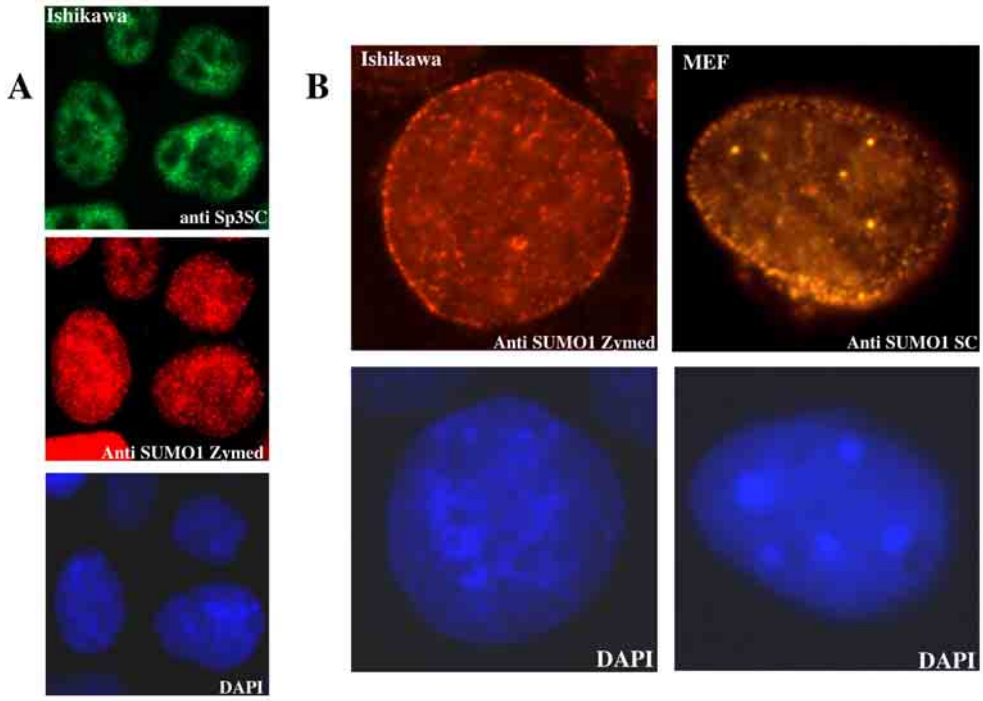


FIG. 3.17. Subcellular localization of endogenous Sp3 and SUMO1.
A. Sp3 was detected by immunostaining with a rabbit anti-Sp3SC antibody and a FITC and SUMO1with anti SUMO1monoclonal and GAM-Texas Red conjugated secondary antibody.
B. Higher magnification of SUMO1 localization in Ishikawa and MEFs cells. In Ishikawa cells, SUMO1 was detected with anti SUMO1 monoclonal and GAM-Texas Red and in with a rabbit anti-SUMO1SC antibody and a Cy3 conjugated secondary antibody. Control staining of nuclei was performed with DAPI.

3.2.3.2. Overexpression of SUMO1 and SUMO2 does NOT Alter Sp3 Subcellular Localization

It was reported that SUMO overexpression may have implications upon subcellular localization of certain substrates such as PML, Sp100, IkB, and p53 by recruiting them into PML-containing nuclear dots (Melchior, 2000).

To address this question the effect of SUMO overexpression on subcellular localization of Sp3 by recruiting it to nuclear bodies was examined. Two different

experiments were performed. Initially, I analyzed the localization of endogenous Sp3 after SUMO1 and SUMO2 overexpression. Subsequently the effect of Ubc9 and Sp3 coexpression was examined.

3.2.3.2.1. SUMO1 and SUMO2 Overexpression

To investigate the subcellular localization of endogenous Sp3 upon SUMO overexpression, I performed indirect-immunofluorescence analyses. Ishikawa cells were transfected with expression constructs for GFP-SUMO1 or GFP-SUMO2. 24 hours post transfection, cells were subjected to immunostaining (see Methods chapter). Endogenous Sp3 localization was detected with a rabbit anti-Sp3 antibody and a GAR-AF-594 secondary antibody and GFP-SUMO1 or GFP-SUMO2 were visualized by GFP intrinsic green fluorescence.

A Bio-Rad confocal system connected to a Nikon inverted microscope designed for the precise separation of fluorophore channels was used. The GFP fluorophore was excited with 488 nm laser line of an Argon-Ion laser. Fluorescence emission was collected through a 500 nm longpass emission filter. AF-594 fluorophore was excited with 543 nm laser line of the HeNe-Ion laser. Fluorescence emission was collected through a 600 nm longpass emission filter.

The confocal technique was chosen to prove unequivocally that Sp3–SUMO localization avoiding fluorophore interference could lead to unspecific colocalization.

Fig.3.18 depicts the subcellular localization of Sp3 in Ishikawa cells after transfection with a GFP- SUMO1 (A) or SUMO2 (B) constructs. Both proteins, Sp3 and SUMO, were localized predominantly within the nucleus. Immunostaining revealed that SUMO1 and SUMO2 have partially overlapping but distinct patterns of localization. However, overexpression of GFP-SUMO1 or GFP-SUMO2 did not alter the localization of Sp3. It should be noted that the high resolution of confocal microscope direct to acquisition of Sp3 and SUMO images somewhat unusual from images taken by using a fluoresce microscope. Confocal Sp3 and SUMO images appear more punctuate for the reason that the confocal resolution (discrimination between two points) is much higher compared to classical microscopes.

In conclusion, there is no similarity between expression pattern of endogenous Sp3 and overexpressed SUMO1 or SUMO2, but the obvious result of this experiment is that the endogenous Sp3 nuclear distribution is not changed following SUMO1 or SUMO2 overexpression.

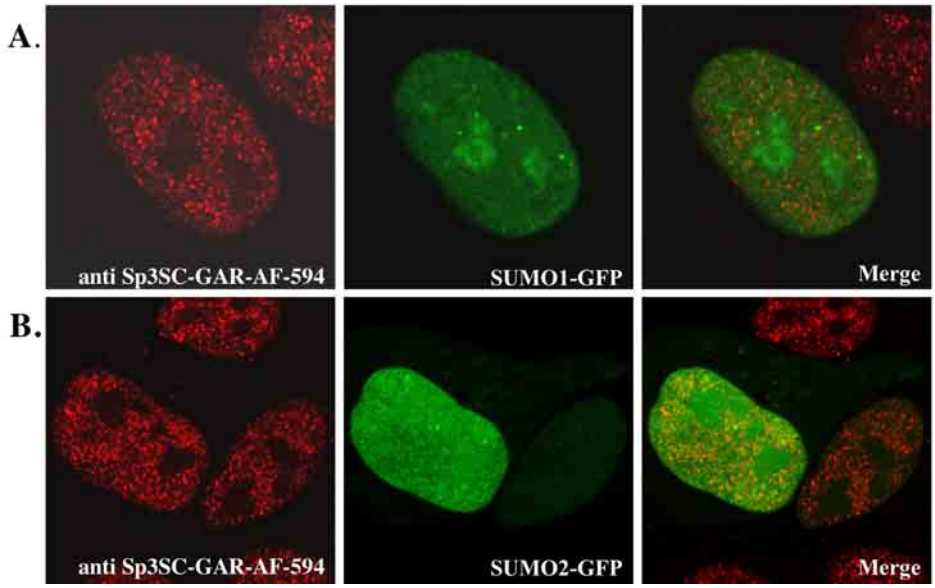


Fig. 3.18. Subcellular localization of endogenous Sp3 after SUMO1 and SUMO2 overexpression.
Ishikawa cells were transfected with 1 μg of an expression construct for GFP-SUMO1 (A) or GFP-SUMO2 (B). Visualization was by the intrinsic green fluorescence of the GFP moiety. Endogenous Sp3 localization was detected with a rabbit anti-Sp3 antibody and a GAR-AF-594 secondary antibody.
The images were recorded with Radiance 2000 confocal system produced by Bio-Rad Cell Science Division (Hemel Hempstead, United Kingdom) adapted to an Eclipse T300 inverted microscope (Nikon Corporation, Tokyo, Japan).

3.2.3.2.2. Ubc9 and Sp3 Coexpression

Ubc9, the conjugation enzyme for SUMO, is predominantly present in the nucleus and at the nuclear pore complex (Schwarz et al., 1998).

In Fig.3.19.A. Ishikawa cells were transiently transfected with expression constructs for GFP-SUMO1 and Ubc9 (described in Materials chapter). Endogenous Sp3 was detected with a rabbit anti-Sp3 antibody and a GAR-AF-594 secondary antibody and GFP-SUMO1 by the built-in green fluorescence of the GFP tag.

In Fig.3.19.B. Ishikawa cells were transiently transfected with expression constructs for GFP-Sp3 and FLAG-SUMO1. SUMO1 was detected with an anti FLAG monoclonal and GAM-Texas Red conjugated secondary antibody and Sp3 by the intrinsic green fluorescence of the GFP.

When Ubc9, in addition to SUMO1, was overexpressed a striking redistribution and accumulation of SUMO1 into punctuated structures was observed, whereas the diffuse distribution of Sp3 was not altered (Fig.3.19.A). After overexpression of Sp3 (GFP-Sp3) and SUMO1 (FLAG-SUMO1) we found that the GFP-Sp3 pattern appears to resemble Sp3 endogenous distribution. Nevertheless, this specific pattern is not affected by SUMO1 overexpression (Fig.3.19.B).

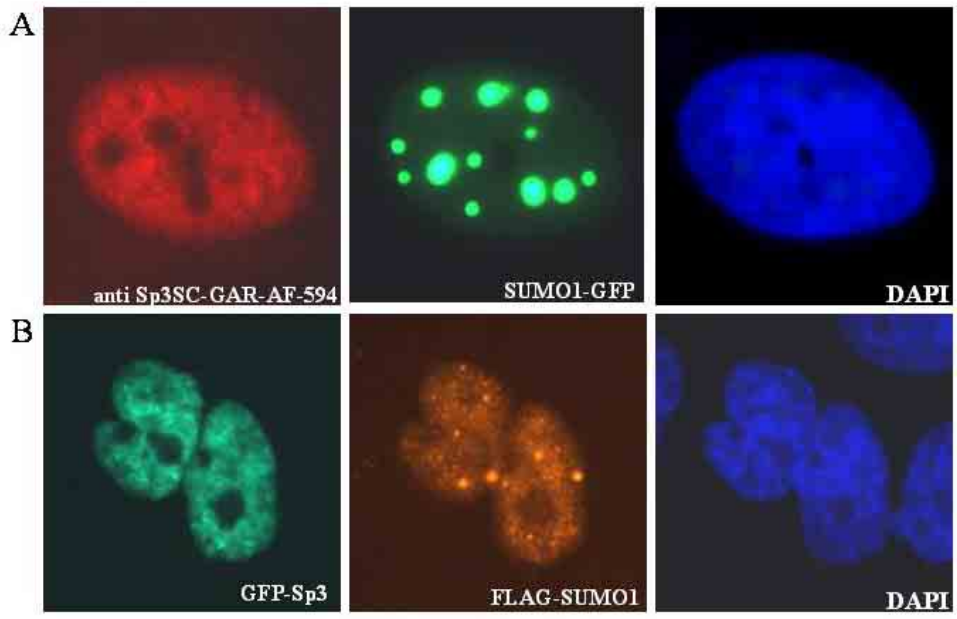


Fig.3.19- A. **Coexpression of SUMO1 and Ubc9 does not alter endogenous Sp3 subcellular localization**. Ishikawa cells were transfected with 1 µg of an expression construct for GFP-SUMO1 and Ubc9. Visualization was by the intrinsic green fluorescence of the GFP moiety. Endogenous Sp3 localization was detected with a rabbit anti-Sp3 antibody and a GAR-AF-594 secondary antibody.
B. **SUMO1 and Sp3 overexpression**. Ishikawa cells were transfected with 1 µg of expression constructs for GFP-Sp3 and FLAG-SUMO1. SUMO1 was detected with anti FLAG monoclonal and GAM-Texas Red conjugated secondary antibody and Sp3 by the intrinsic green fluorescence of the GFP moiety.

Given that SUMOylation at the IKEE motif of the ID is essential for silencing the transcriptional activation capacity of Sp3, one could speculate that SUMOylated Sp3 alters the interaction with other nuclear proteins, which in turn alters the sub-nuclear distribution of Sp3. Thus far, we have no indication for such a mechanism. A diffuse nuclear distribution of Sp3 was observed even under conditions where a dramatic accumulation of SUMO1 in punctuate nuclear structures occurred and even after coexpression of Ubc9.

Taken together, these immunofluorescence experiments reveal that Sp3 is not targeted to nuclear bodies even under conditions where SUMO1 becomes predominantly accumulated in such structures.

3.2.3.3. Recruitment of Sp3 to Nuclear Speckles is an Artifact

The mammalian cell nucleus contains a large number of nuclear bodies that are thought to function in a variety of nuclear processes. One of the most prominent nuclear structures is the promyelocytic leukemia (PML) nuclear body, also known as PML oncogenic domain, nuclear domain 10, or Kremer body. There are usually between 5 and 30 PML bodies per nucleus, depending on cell type and stage of the cell cycle (Koken et al., 1995).

So far, almost all SUMO targets are influenced at subcellular localization level by SUMOylation in one way or another (Wang et al., 2004). Data presented in the previous chapter indicates that Sp3 localization is not affected by endogenous SUMO or overexpression of SUMO. However, under certain conditions Sp3 was redistributed after SUMO overexpression, but it was unclear whether Sp3 accumulation in dot-like structure was specific or not.

To determine if this localization was specific, Ishikawa cells were cotransfected with expression constructs for GFP-SUMO1 and Ubc9. SUMO1 was detected by the intrinsic green fluorescence of GFP and endogenous Sp3 localization was detected with a rabbit anti-Sp3 antibody and a GAR-CY3 secondary antibody.

At first glance, the Sp3 localization pattern (Fig.3.20-A) was not affected by transfected SUMO1 (Fig.3.20-B). However, if Sp3 localization signal was recorded

following GFP observation “a striking accumulation of Sp3 in dot-like structure” was observed (Fig.3.20-C). This phenomenon can occur when the emission spectrum of a donor fluorophore (GFP) significantly overlaps the absorption spectrum of an acceptor - CY3 Fig.3.20-E) and the energy from one fluorophore (‘Donor’) is transferred to a different fluorophore (‘Acceptor’).

We found Sp3 recruitment in nuclear dots only under special conditions when fluorescence signal of GFP-SUMO1 additionally excited the Sp3 conjugated fluorophore (Fig.3.20-C).

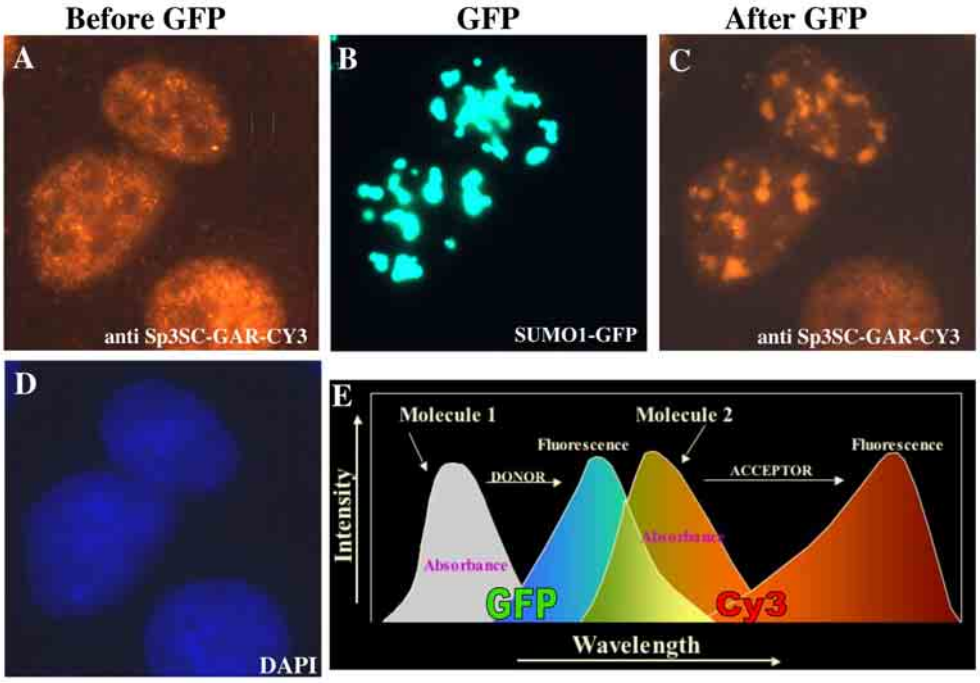


Fig.3.20. Sp3 and dot-like structures in Ishikawa cells.
A. Subcellular localization of endogenous Sp3 detected with a rabbit anti-Sp3 antibody and a GAR-CY3 secondary antibody. **B.** Overexpressed SUMO1 visualized by the intrinsic green fluorescence of the GFP moiety. **C.** When emission spectra (fluorescence) of the GFP fluorophore is overlapping absorbance spectra of another fluorophore (Cy3), the intensity of Cy3 is increased and produces dramatically changes in Sp3 localization pattern. **D.** Control staining of nuclei was performed with DAPI.
E. Graphic explanation of the energy transfer from ‘Donor’ fluorophore (GFP) to a different fluorophore, CY3 (‘Acceptor’).

3.3. Is Sp3 Regulated by SUMOylation?

3.3.1. Sp3 SUMOylation in Different Cell Lines and Tissues

In the first chapter of results it was shown that Sp3 is expressed as four distinct proteins, two slow migrating of more than 100 kDa and two fast migrating species of approximately 72 kDa that differ in the extent of the amino terminal part. All four isoforms become SUMO-modified in vivo specifically and exclusively at lysine residue 551.

Based on these findings, I wanted to know whether the relative amount of the Sp3 isoforms as well as the Sp3-SUMO modification varies in different cell lines.

In order to solve this question, I used several tumor cell lines of human and mouse origin, grown as a monolayer in cell type specific culture conditions. It was necessary to opt for cells derived from endometrial tissue (Ishikawa cells), kidney (Phoenix cells), colon (CaCo-2 cells and SW620 cells), epithelium (HeLa cells), keratinocyte (HaCat cells), bone (Saos-2 cells), neuroblastoma (IMR32 cells) or mouse fibroblasts (NIH3T3 cells). The cell lines were grown in specific media conditions (see Materials chapter) and lysed in SDS containing buffer as soon as the cells become confluent. Equal amount of proteins from fresh SDS cell extracts were used for immunoprecipitation with anti Sp3 specific antibody and were separated through 6 % PAGE.

As can be seen in Fig.3.21, the Sp3 protein is expressed in all cell lines analyzed. The amount of Sp3 is slightly different between cell lines but the Sp3 SUMOylation remains unchanged. Two different exposures (Fig.3.21 A for 10 min and Fig.3.21 B for 3 min) were shown in order to permit better observation of Sp3 expression and SUMO modification. All four Sp3 isoforms are expressed and are SUMOylated. The ratio between isoforms is not changed and the percentage of SUMO modification does not vary (Fig.3.21).

The Sp3 expression analysis in various cancer cell lines, derived from different organs upon immortalization, did not reveal alterations in isoforms ratio and more important, SUMOylation level of Sp3 appears to be not cell type dependent.

The next question was whether the SUMO modification of Sp3 is varies in different organs.

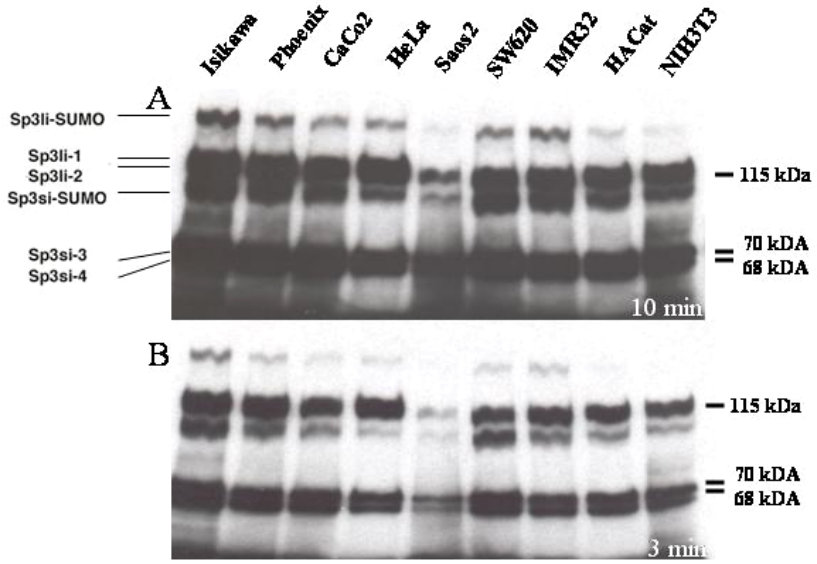


Fig.3.21. Non-modified and SUMO-modified Sp3 protein in different cell lines. Western blot analyses of Sp3 in different cell lines. Cells were lysed with SDS-containing buffer. Equal amounts of protein (100 µg) from cell extracts were subjected to IP with anti-Sp3SC antibodies and subsequent separated through 6% SDS polyacrylamide gels. After blotting, PVDF membrane was incubated with Sp3-specific antibodies.
A and B- different exposure times are shown.
Abbreviations are: Sp3li-1 and 2, long isoforms of Sp3; Sp3si-3 and 4, small isoforms of Sp3; Sp3li-SUMO and Sp3si-SUMO, SUMO-modified long and small isoforms of Sp3.

To get a straightforward answer to this question, the Sp3 expression in organs prelevated from mice were analyzed. Mice were killed in agreement with the regulations of the local animal welfare committee. The organs were prelevated in ice. After weighing, organs were mixed with 5 fold excess of SDS lysis buffer and homogenized with a Potter device at variable speed. Homogenized organs were boiled for 10 min and centrifuged for 10 to 15 min until complete clearing up of the supernatant. Equal amounts of protein from cell extracts were subjected to immunoprecipitation with anti Sp3 antibody and separated through 6% PAGE.

Immunoblotting was performed with the same commercially available anti Sp3 antibody from Santa Cruz.

In Fig.3.22, the Sp3 expression and SUMOylation pattern is shown in various mouse organs. Although an equal amount of protein (150 µg) from organ extracts was used for immunoprecipitation, the total amount of Sp3 seems to vary. For instance, there is less Sp3 in brain and muscle compared to lung or spleen.

Another observation is that the ratio between Sp3 unmodified and SUMOylated Sp3 is not significantly changed. For a better comparison, two different exposure times of the immunoblot are shown (Fig.3.22 A for 10 min and Fig.3.22 B for 3 min).

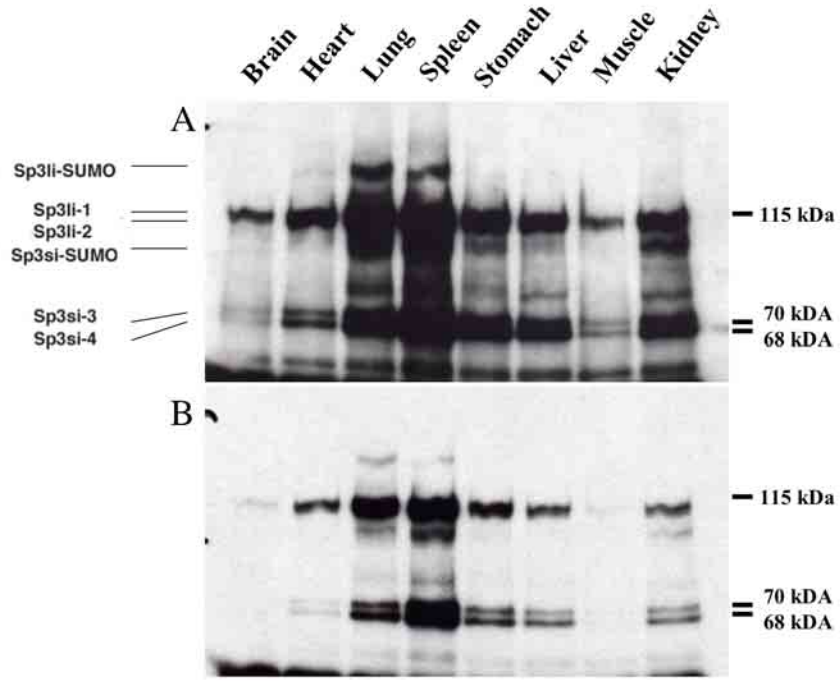


FIG 3.22. Endogenous Sp3 protein expression and SUMOylation level in different mouse organs. Western blot analyses of Sp3 in organs indicated above. Different organs were prelevated from adult mice and homogenized in SDS-containing buffer. Equal amount of proteins (150 µg) from organ extracts were used for IP with anti-Sp3SC antibodies and then separated through 6% SDS polyacrylamide gels. After blotting, PVDF membrane was incubated with Sp3-specific antibodies.
A and B- different exposure times of the PVDF membrane.
Abbreviations are: Sp3li-1 and 2, long isoforms of Sp3; Sp3si-3 and 4, small isoforms of Sp3; Sp3li-SUMO and Sp3si-SUMO, SUMO-modified long and small isoforms of Sp3.

The Sp3 protein is expressed in all cell lines and in all mouse organs analyzed. All four isoforms were detected without shifting the expression ratio between long and small isoforms. More importantly, the Sp3 SUMOylation does not point up substantial differences among various unrelated cell types and tissues investigated. One can conclude that the level of Sp3 modification by SUMO is not cell line or organ dependent.

3.3.2. Is SUMOylation of Sp3 Regulated by Different Stress Conditions?

Different cells in an individual contain the same genome, but the same cells show a different gene expression pattern, even if those cells are of the same cell type. Cells can quickly change their expression pattern in response to a plethora of changes in their environment, like temperature, light conditions, redox potential, and nutrient supply or in response to external signaling molecules like hormones, growth factors and cytokines.

The previous experiments demonstrated that SUMOylation of Sp3 is not significantly modulated upon SUMO1 or SUMO2 overexpression and the level of Sp3 modification is not cell type or tissue dependent.

Another question was whether Sp3 modification by SUMO is regulated by different stress conditions. To decipher this question, I performed further experiments in order to find out if the level of Sp3 SUMOylation may change under different metabolic stress conditions or after treatment with drugs known to play important roles in some regulatory pathways.

3.3.2.1. Serum Induction, Serum Starvation and Heat Shock

I started with serum induction and starvation of several cell lines and also with heat shock treatment in Ishikawa, NIH3T3 and Saos-2 cells.

All cell lines were grown in recommended medium containing 10% fetal calf serum (FCS) in 9 cm Ø culture dishes until 70-80% confluence. The cells were washed three times with PBS and the medium was replaced with medium without FCS for

periods of time indicated in the Fig.3.23. Cells subjected to heat shock were placed into cell culture incubator at 42 ^{0}C in the presence of 5% CO_2. As a control, cells grown in normal media were used. In order to preserve SUMOylation, SDS cell extracts were prepared and protein concentration determination was performed. Equal amounts of protein were separated through 6% PAGE and after blotting, the membranes were incubated with anti Sp3 antibody and anti Sp1 serum, respectively.

The SUMOylation level of Sp3 was influenced by diverse experimental conditions in various cell lines as can be seen in Fig.3.23. Even when the cells were incubated for a long time with medium in the absence of FCS (the first two lanes of each blot: - 24hFCS and -21hFCS +3hFCS), the total amount of Sp3 was reduced but the ratio between Sp3 and Sp3-SUMO obviously is not changed. This ratio is not distorted after short term starvation (+21hFCS -3hFCS) and heat shock (3h at 42 ^{0}C).

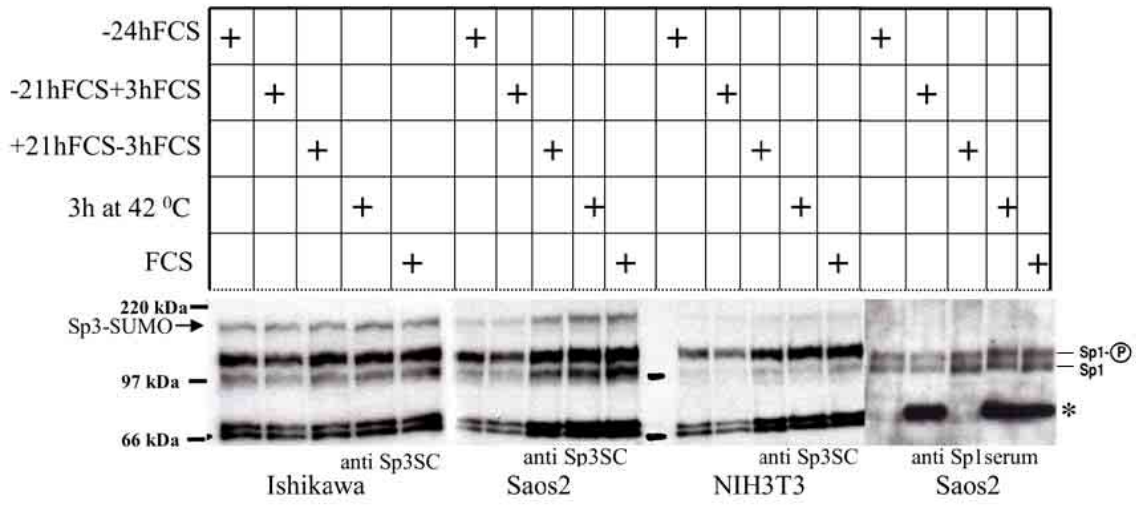


Fig.3.23. SUMO-modification of Sp3 after various stress conditions. Ishikawa, Saos2 and NIH3T3 cells were grown in the absence of fetal calf serum in medium for 24 hours (-24hFCS), in the absence of FCS for 21 hours and the last 3 hours in normal medium (-21hFCS+3hFCS), in normal medium for 21hours and then 3 hours in medium without FCS (+21hFCS-3hFCS), in normal medium and 3 hours incubated at 42 ^{0}Celsius (3h at 42 ^{0}C) and control cells in normal media (FCS). Cells were lysed with SDS-containing buffer and then separated through 6% SDS polyacrylamide gels. After blotting, PVDF membrane was incubated Sp3 or Sp1-specific antibodies as indicated. The star marks the IgG from FCS.

As well, the expression pattern of Sp1 is not influenced by these experimental conditions: the ratio between Sp1 and the Sp1 phosphorylated form remained invariable. It can be noticed that the band marked with star (*) in the anti Sp1 blot, disappears in starved cells. Most likely, the band is due to the recognition of immunoglobulins from FCS by anti Sp1 serum.

As seen in Fig.3.23, there is no variation in Sp3 expression pattern after serum starvation, serum induction and heat shock for three hours. It also can be possible that SUMOylation is a very fast event and can occur in a short period of time. To elucidate this aspect, I performed additional experiments using a short serum starvation period (Fig.3.24) and induction (Fig.3.25) in SaOs-2 cells. These osteosarcoma cells were used as Sp3 KO mice display bones and teeth with impaired development (Bouwman et al., 2000).

A. Short Period Serum Starvation.

The experimental conditions were similar to the previous experiment. For serum induction, Saos2 cells were grown in medium containing 10% fetal calf serum (FCS) in 9 cm Ø culture dishes for 48 hours. After intensive washing with PBS, the medium was replaced with medium without FCS for periods of time indicated in Fig.3.24.

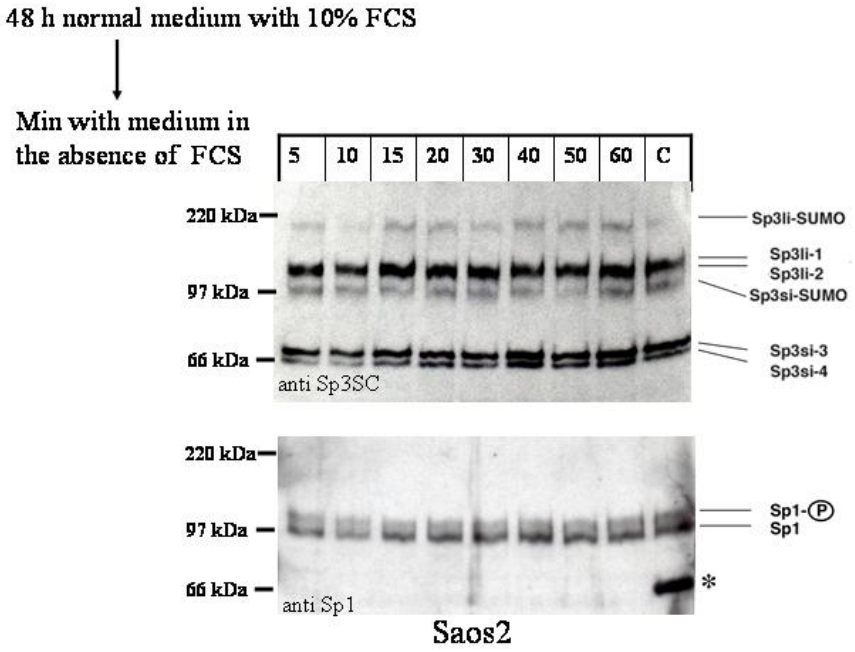


Fig.3.24. SUMOylation pattern of endogenous Sp3 after serum starvation. Saos2 cells were grown in normal medium for 48 and then, in the absence of FCS for 5-60

minutes as indicated. Cells were lysed with SDS-containing buffer. Equal amounts of protein (20 µg) were separated through 6% SDS polyacrylamide gels. After blotting, PVDF membrane was incubated Sp3 or Sp1-specific antibodies as indicated. The star indicates the IgG from FCS.

B. Short Period Serum Induction.

For the short period serum induction experiments, the Saos2 cell were grown in medium containing 10% fetal calf serum (FCS) in 9 cm Ø culture dishes until 70-80% confluence and then the medium was replaced with serum-free medium for 24 hours. This medium was substituted subsequently with normal medium including 10% FCS for time points indicated in Fig.3.25.

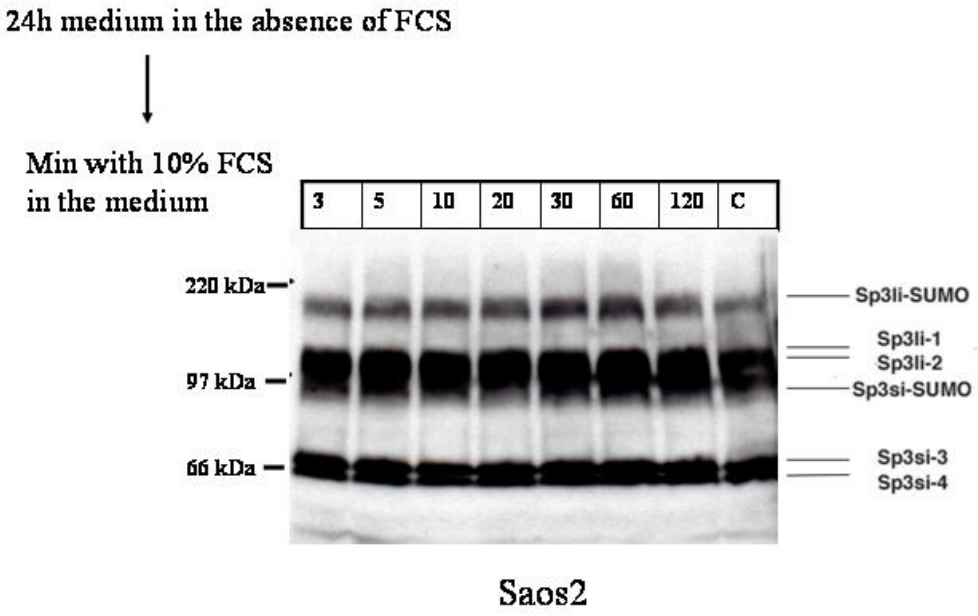


Fig.3.25. SUMOylation pattern of endogenous Sp3 after serum induction.
Saos2 cells were grown in medium without FCS for 24h and then, in normal medium containing 10% FCS for 3-120 minutes as indicated. Cells were lysed with SDS-containing buffer and 20 µg of proteins were separated through 6% SDS polyacrylamide gel. After blotting, the PVDF membrane was incubated with a Sp3 -specific antibody.

One can conclude that the experimental conditions illustrated in Fig.3.23, Fig.3.24 and Fig.3.25, respective serum induction, serum starvation and heat shock does not

alter Sp3 SUMOylation. Also the Sp1 expression and phosphorylation status is not influenced by the metabolic stress conditions applied to mammalian cells in our experiments.

3.3.2.2. Ultraviolet Radiation, TNF-alpha and MG132

Exposure of cells to DNA-damaging agents elicits a complex set of acute cellular responses. Generally, DNA damage-inducible (DDI) responses serve to protect the cell from genotoxic adversity. The most studied component of mammalian DDI responses is the "Guardian of the Genome" p53 tumor suppressor gene product. p53 becomes activated by genotoxic and other stresses, and in turn p53 activates transcription of perhaps as many as 100 other genes. Indeed, treatment of cells with UV radiation and other DNA-damaging agents was effective in activating p53 as a transcription factor and in turn up-regulating its downstream effectors genes, including p21Waf1/Cip1/Sdi1 (Vogelstein et al., 2000).

We wanted to know whether UV radiation may influence Sp3 SUMOylation status. HEK293 cells were exposed to UV radiation at time periods indicated in the Fig.3.26 A. The medium was partially removed in order to allow the UV to reach the cells but to keep cells humid, knowing that the penetrability of short wave radiations is reduced compared with long wave radiations (IR for example). I used a UV Bench Lamp having two 15 watt long wave bulbs with ensuing technical characteristics: at 30 cm distance from source, the light intensity is 730 Microwatts / cm^2. The wavelength range is 315-400 nm with the peak at 360 nm.

After exposing HEK293 cells with an elevated dose of UV radiation, I did not observe an alteration in Sp3 SUMOylation. Even after one hour of UV exposure when most of the cells appear to be morphologically affected, the Sp3 expression pattern did not change (Fig.3.26 A).

In order to uncover the conditions in which the SUMOylation of Sp3 is influenced, I continued to test the effect of two drugs upon this modification, Tumor Necrosis Factor alpha and MG-132.

TNF-alpha is a pleiotrophic pro-inflammatory cytokine secreted by various cells including adipocytes, activated monocytes, macrophages, B cells, T cells and fibroblasts. It belongs to the TNF family of ligands and signals through two receptors,

TNFR1 and TNFR2. Binding of TNF-α to the receptor can promote either cell survival or death, depending on the context. TNF-α can promote a variety of cellular responses including the recruitment and activation of Caspase-8 in the receptor complex, which initiates a pathway to implement cell death by apoptosis and cell survival by activating the transcription factor NF-kB and the JNK pathway, which together activate anti-apoptotic genes (Ferri et al., 2001). In general, if expression of these genes is suppressed (e.g., by the protein synthesis inhibitor cycloheximide) apoptosis becomes the default response of the cell. TNF-alpha is cytotoxic to a wide variety of tumor cells and is an essential factor in mediating the immune response against bacterial infections. TNF-alpha also plays a role in the induction of septic shock, auto immune diseases, rheumatoid arthritis, inflammation, and diabetes (Yu et al., 2004).

HEK293 cells were treated with 50 ng/ml TNF-alpha, 10 μg/ml cycloheximide or both together, according to dosage recommendations of researchers from our Institute (who use those drugs currently for apoptosis investigation). TNF-alpha treatment did not lead to an alteration in Sp3 SUMOylation pattern. It can be observed that cycloheximide treatment reduces the Sp3 expression. Nevertheless, there is no detectable alteration in SUMOylation pattern in cells treated with both, cycloheximide and TNF-alpha drugs (Fig.3.26 B).

MG-132 - N^{α}-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal [Z-Leu-Leu-Leu-H] a leupeptin analogue is a cell-permeable inhibitor of the proteasome that has been shown to induce apoptosis in MOLT-4 and L5178Y cells *via* a p53-dependent pathway1 and also to enhance CPP32- like activity in TNF-treated U937 cells (Fujita et al., 1996).

I tested this proteasome inhibitor drug by treating HEK293 cells with 25 μM MG-132 for 24 h. The SDS cell extracts from cells treated with and without MG-132 were prepared and Sp3 was immunoprecipitated by anti Sp3 antibody.

I observed an alteration of endogenous Sp3 SUMOylation (Fig.3.26 C). After MG-132 treatment, the intensity of SUMO bands in WB is dramatically reduced.

I also used the immunoprecipitated Sp3 from cells treated with this drug for Western blot detection of ubiquitination of Sp3 by using polyclonal anti Ubiquitin antibody from Sigma, but unsuccessfully.

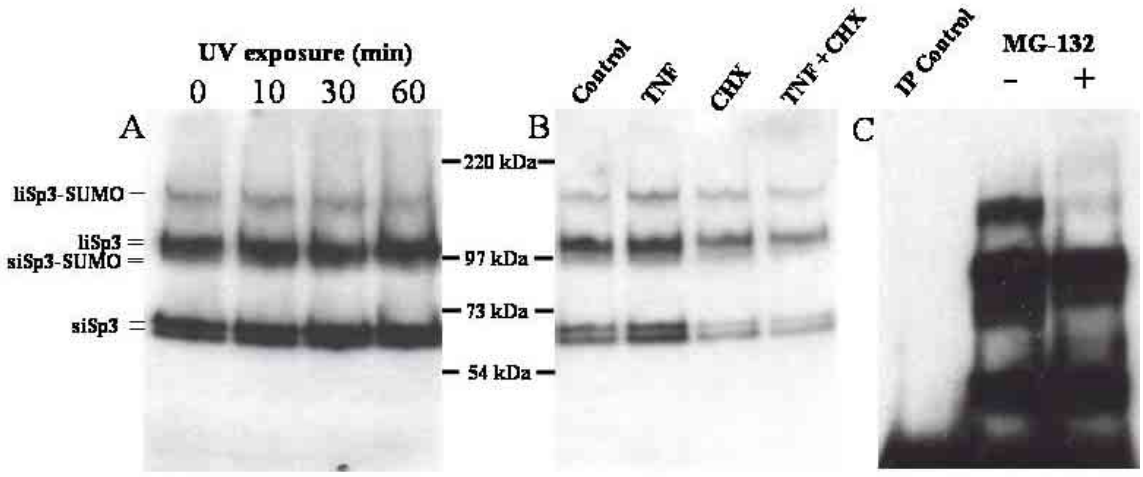


Fig. 3.26. Sp3 SUMOylation after UV and drugs treatment.
A. HEK293 cells were exposed to UV radiation at time periods indicated above figure. Cells were lysed with SDS-containing buffer.
B. HEK293 cells were treated with (50 ng/ml) TNF-alpha (TNF), 10μg/ml cycloheximide (CHX) or both (TNF+CHX). Control cells are nontreated. Cells were lysed with SDS-containing buffer.
C. HEK293 cells were treated with 25 μM MG 132 for 24 h. Cells were lysed with SDS-containing buffer and subjected to IP with anti Sp3SC antibody and no antibody for IP Control.
The proteins were separated through 6% SDS polyacrylamide gels. After blotting, PVDF membrane was incubated with anti Sp3 antibody.

UV radiation or TNF alpha and cycloheximide treatment of mammalian cells did not alter the SUMOylation level of Sp3 protein in our experimental conditions. A significant reduction in Sp3 SUMO modification is observed upon MG-132 treatment. There are many causes that could provoke this alteration. Possibly this proteasome inhibitor is implicated in preventing proteasome degradation of SUMO specific proteases, which subsequently cut off the Sp3-SUMO moiety.

3.3.2.3. Trichostatin A

It has been known for some time that lysine residues in the N-terminal tails of histones can be covalently modified by acetylation, which leads to transcriptionally active chromatin. More recently it became evident that in addition to histones, other nuclear proteins can also be targets of acetylation events. Among them, Sp3 was found to be acetylated in vivo (Braun et al, 2001).

Histone deacetylase (HDAC) inhibitors have shown significant anti-proliferative and apoptotic properties on various cancer cells, including prostate cancer, and are therefore being evaluated as treatment modalities. Among them, three structurally unrelated HDAC inhibitors, trichostatin A (TSA), depsipeptide (FR901228), and sodium butyrate are preferentially used in most of the publications.

In our lab it was shown that Sp3 is acetylated in vivo (after TSA treatment). A mutant of Sp3 lacking the K551 SUMO target residue exhibits a far weaker acetylation compared with wild type (Braun et al., 2001).

The purpose of our experiment is to test whether the TSA treatment may influence Sp3 SUMOylation in order to determine the connection between this modification and acetylation.

To address this question, I transfected different Sp3 expression constructs (as shown in Fig.3.27), wt construct and three others containing point mutations in the SUMOylation site, into SL2 insect cell lines. In order to enhance the Western blot signal of transfected constructs, I performed immunoprecipitations by using anti Sp3 specific antibody as can be seen in Fig.3.27 shorter exposure time of Western blot membrane (1 min).

The Sp3 SUMO modification is detected only in the wt construct emphasizing that the integrity of the SUMO consensus motif is critical for SUMOylation. But most important, the TSA treatment of insect cells does not affect the SUMOylation level of transiently transfected Sp3.

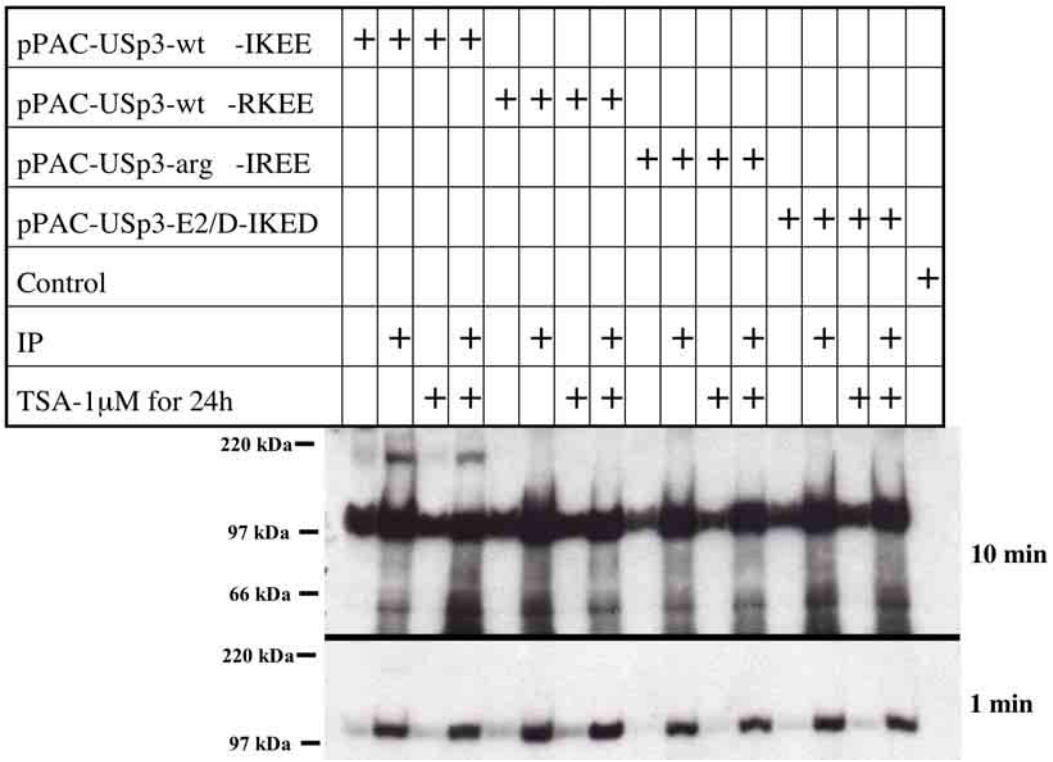


Fig.3.27. Sp3 and corresponding SUMOylation motif mutants expressed in SL2 cells in the presence or absence of TSA.
SL2 cells were transfected with Sp3 expression constructs wt or mutants and treated with 1 μM TrichostatinA (TSA) for 24 h as indicated. Cells were lysed with SDS-containing buffer and then subjected to IP with anti Sp3SC antibody. Cell extracts and IP proteins were separated through 6% SDS polyacrylamide gels.
After blotting, PVDF membrane was incubated with Sp3 -specific antibody and two exposure times are shown (1min and 10 min).

3.4. PIAS1- Nuclear Protein and E3 Ligase

3.4.1. PIAS1 is Located into Nucleus

Modification of proteins by SUMO is a three-step process that requires three classes of enzymes, E1–E3. An E1 activating enzyme and E2 conjugating enzyme prime SUMO for ligation to a substrate by an E3 SUMO ligase. There are several E3 ligases that are responsible for transferring activated SUMO to their substrates. For exemple Polycomb Protein Pc2, and nucleoporin RanBP2, which has been previously

characterized for its function in nuclear transport and RING-domain containing proteins of the protein inhibitors of activated STATs (PIAS) family, have been identified as E3 ligase enzymes for SUMO1 (Hilgarth et al., 2004).

An important mechanism to control the activity of transcriptional regulators appears to be their targeting to specific subnuclear sites. Accumulating evidence indicates that the PIAS/SUMO system is involved in this process.

We wanted to know whether PIAS1 displays a particular localization pattern and whether PIAS1 affects Sp3 specific nuclear distribution.

To address this question, immunofluorescence experiments were performed in HEK293 cells. Firstly, I did coimmunostaining for endogenous PIAS1 and Sp3. Sp3 was immunostained with a rabbit anti-Sp3SC (Santa Cruz) antibody and a (Goat Anti Rabbit) GAR-FITC conjugated secondary antibody. PIAS1 was detected with anti PIAS1 goat and (Donkey Anti GOAT) DAG-AF594 conjugated secondary antibody (Fig.3.28.A). The same antibody combinations were used to detect overexpressed PIAS1 and Sp3 in HEK293 cells transiently transfected with an untagged PIAS1 expression construct –pN3- SUMO1 (Fig.3.28.B).

To observe the influence of overexpressed SUMO1 and PIAS1 on Sp3 localization, I cotransfected expression constructs for PIAS1 (pN3-PIAS1) and SUMO1 (GFP-SUMO1). Sp3 was detected by immunostaining with a rabbit anti-Sp3SC antibody and a GAR-AF594 secondary antibody. Detection of SUMO1 was achieved by intrinsic green fluorescence of the GFP moiety (Fig.3.28 -C).

For PIAS1 subcellular localization control, I cloned PIAS1 full-length sequence as a GFP fusion into the MCS of pGFP-N3 (Clontech). HEK293 cells were transfected with the new construct-pGFP-N3-PIAS1. In living cells, PIAS1 was visualized by the fluorescence of GFP and control cell detection by phase contrast (Fig.3.28 -D).

PIAS1 was found to be located in the nucleus and displayed a similar localization pattern as Sp transcription factors (Fig.3.28.A and D). After overexpression, some of the PIAS1 protein was recruited into nuclear dots (Fig.3.28.B). This recruitment did not affect subcellular localization of Sp3 even when SUMO1 was overexpressed with PIAS1 (Fig.3.28.C).

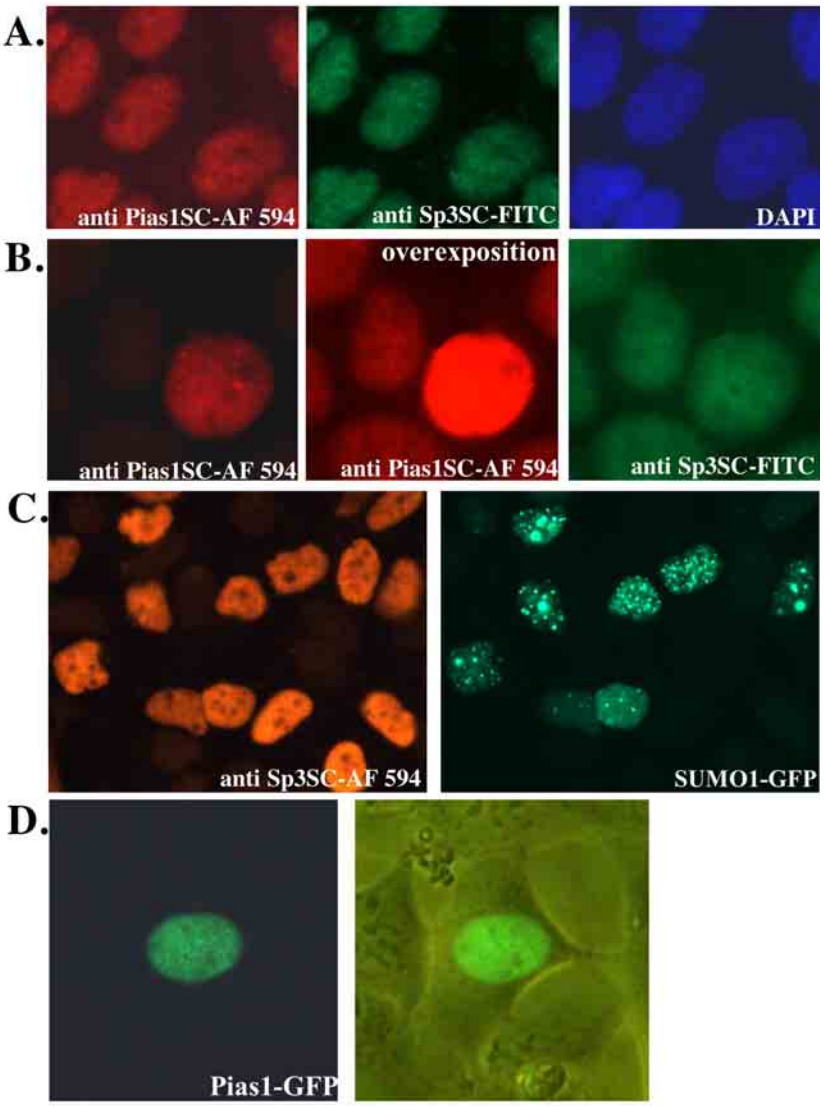


FIG.3.28. Subcellular localization of PIAS1 in HEK293 cells.

A. Endogenous PIAS1 and Sp3. Sp3 was detected by immunostaining with a rabbit anti-Sp3SC antibody and a FITC conjugated secondary antibody. Detection of PIAS1 was with anti PIAS1SC goat and DAG-AF594 conjugated secondary antibody. Control staining of nuclei was performed with DAPI.

B. HEK293 cells were transfected with an expression construct for PIAS1 (pN3-PIAS1). Sp3 was detected by immunostaining with a rabbit anti-Sp3SC antibody and FITC, and PIAS1 with anti PIAS1 goat and DAG-AF594 conjugated secondary antibody.

C. HEK293 cells were transfected with expression constructs for PIAS1 (pN3-PIAS1) and GFP-SUMO1. Sp3 was detected by immunostaining with a rabbit anti-Sp3SC antibody and an AF594 and SUMO1 by the intrinsic green fluorescence of GFP moiety.

D. HEK293 cells were transfected with GFP-PIAS1 (pEGFP-N3-PIAS1) Visualization was by the intrinsic green fluorescence of GFP moiety. In the right figure is shown the entire transfected cell by phase contrast.

In conclusion, endogenous PIAS1 displayed a diffuse nuclear localization pattern similar to the Sp3 transcription factor. After overexpression, one observed a redistribution of PIAS1 signal to nuclear dots, that did not affect the Sp3 pattern. When PIAS1 was cotransfected with SUMO1, the GFP-SUMO1 signal was enhanced in dot-like structures. A lower magnification picture of PIAS1 along with SUMO1-GFP cotransfection experiment was exposed (Fig.3.28.C), which demonstrated GFP-SUMO1 dots in different cells were variable. In some cells, one observed diffuse nuclear distribution of GFP-SUMO1 and few dots.

In other cells, SUMO1-GFP exhibited microgranular localization in small foci, or was concentrated in fewer, but larger granules. It is important to notice that none of this particular SUMO1 pattern observed after PIAS1 coexpression had any influence on Sp3 subcellular localization.

3.4.2. PIAS1 is a SUMO E3 Ligase

Protein inhibitors of activated STATs (PIAS) proteins were initially identified as negative regulators of cytokine signaling that inhibit the activity of STAT-transcription factors. Evidence that illustrates PIAS proteins function as transcriptional coregulators in various other important cellular pathways, including Wnt signaling, the p53 pathway and steroid hormone signaling (Goss et al., 2003) is rapidly accumulating. Recent work from several laboratories has revealed that PIAS proteins act as E3-like ligases that stimulate the attachment of the SUMO to target proteins. Since in most cases the SUMO ligase activity and the transcriptional coregulator activity are functionally correlated, the PIAS/SUMO pathway appears to be an important mechanism of transcriptional regulation (Wormald and Hilton., 2004).

It was demonstrated in our lab that PIAS1 fulfils all requirements to act as an E3 ligase for SUMO conjugation to Sp3, *in vitro*. It interacts with the E2 enzyme Ubc9 and with Sp3 and most importantly, it strongly stimulates transfer of SUMO1 and SUMO2 to Sp3 (Sapetschnig et al 2002).

3.4.2.1 E3 ligase Activity *in vitro*

Recombinant HA/FLAG-tagged Sp3 was subjected to SUMO modification *in vitro* (Sapetschnig et al, 2002) with limiting amounts of recombinant E1 (Aos1 and Uba2 subunits), E2 (Ubc9) enzymes and recombinant SUMO1 ((Fig.3.29.A). Addition of bacterially expressed GST-PIAS1 increased the conjugation of SUMO1 to Sp3 (Fig.3.29.B).

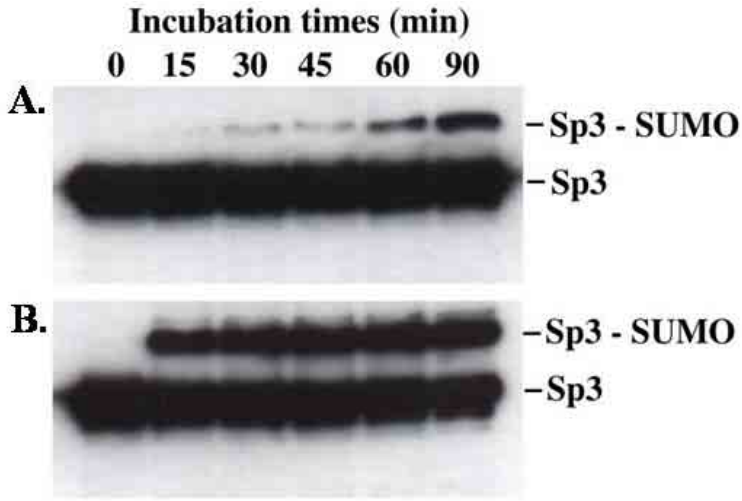


Fig.3.29. E3 ligase activity of PIAS1 in vitro.
A. Recombinant HA/FLAG-tagged Sp3 expressed in SL2 cells, was subjected to SUMO modification in vitro with limiting amounts of SUMOylation mix (recombinant E1 (Aos1 and Uba2 subunits), E2 (Ubc9) enzymes and recombinant SUMO1).
B. Addition of bacterially expressed GST-PIAS1 to SUMOylation mix positively influences the conjugation of SUMO1 to Sp3.
Aliquots of SUMOylation reactions were sampled after zero, 15 30, 45, 60 and 90 minutes of incubation at 30 C. The SUMOylation reaction was stopped by mixing with 2x Laemmli buffer and subsequently, the proteins were separated through 8% SDS polyacrylamide gel. After blotting, PVDF membranes were incubated with Sp3 SC specific antibody.

3.4.2.2. E3 ligase Activity *in vivo*

The next question was whether PIAS1 could enhance SUMOylation *in vivo* following overexpression.

For this purpose, expression constructs for PIAS1 and SUMO1 were transfected into HEK293 cells (Fig. 4.27). 36 hours posttransfection, SDS cell extracts were prepared. The same amount of protein from cell extracts was separated by SDS polyacrylamide gel electrophoresis. After blotting, PVDF membrane was incubated with anti-PIAS1-specific antibody[1], then stripped and blotted with anti-Sp3SC as indicated (Fig.3.30).

Overexpression of PIAS1 protein in HEK293 cells did not enhance endogenous levels of Sp3 SUMOylation. Coexpression of PIAS1 and GFP-SUMO1 did not lead to an enhancement Sp3 SUMOylation.

Even in conditions in which PIAS1 was expressed at extremely high-levels compared to endogenous PIAS1 (Fig.3.30 lower blot probed with anti PIAS1 antibody), the Sp3 expression pattern was not altered.

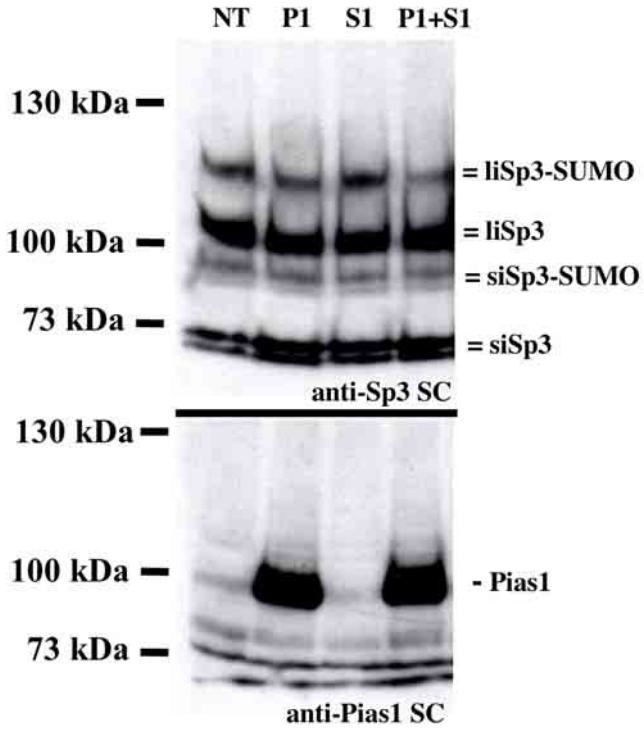


Fig.3.30. Overexpression of PIAS1 does not influence the sumoylation level of Sp3. HEK293 cells were transfected with expression constructs for PIAS1 (pN3-PIAS1, **P1**), SUMO1 (pEGFP-SUMO1, **S1**) or both (**P1 +S1**). **NT** are nontransfected control cells. Cells were lysed with SDS-containing buffer. The same amount of protein from cell

[1] Pias1 detection with anti-PIAS1 antibody was possible at the end of my work due to a change in the blocking step. Blocking with 1% BSA instead 5% Skimmilk was used.

extracts was separated through 6% SDS polyacrylamide gels. After blotting the PVDF membrane was incubated with PIAS1-specific antibody, then stripped and blotted with anti-Sp3SC as indicated.
Abbreviations are: Sp3li-1 and 2, long isoforms of Sp3; Sp3si-3 and 4, small isoforms of Sp3; Sp3li-SUMO and Sp3si-SUMO, SUMO-modified long and small isoforms of Sp3.

3.4.3. Strategies for PIAS1 Protein Complex Purification

Work by several laboratories has showed that mammalian PIAS proteins exert SUMO-ligase activity towards various mammalian SUMO-target proteins.

Even though Ubc9 binds to the SUMO acceptor site and efficiently transfers SUMO to selected targets *in vitro*, a specific SUMO E3-ligating enzyme might be required for efficient and properly targeted modification. E3 ligase activities towards SUMO have been identified recently for members of the PIAS family of proteins (Kahyo et al., 2001; Sachdev et al., 2001; Sapetschnig et al., 2002).

Preliminary nuclear extract fractionation studies suggest that PIAS1 is part of (a) high molecular weight complex(es) *in vivo*. PIAS1-associated proteins might confer substrate specificity towards Sp3 and other transcription factors and/or regulate PIAS1 activity *in vivo*. We suspect the identification of PIAS1-associated proteins to be an important step towards understanding PIAS1 protein functions and specificities *in vivo.*

One obvious question was whether PIAS1 can form such complexes and whether the components of this complex can be identified. The main goal of our work was the cloning and subsequent characterization of PIAS1-associated proteins. As a prerequisite for the purification and identification of PIAS1-associated proteins, establishing a two-step purification method for epitope-tagged PIAS1 was required.

3.4.3.1. Principle of Tandem Affinity Purification

Proteome analysis, in particular using mass spectrometry (MS), requires fast and reliable methods of protein purification. The method most suitable for standardization is affinity purification based on the fusion of a tag, usually a peptide or a small protein,

to the target protein.

The so-called tandem affinity purification (TAP) method (Rigaut et al., 1999) can be employed to allow a two-step purification of appropriately tagged PIAS1. Briefly, PIAS1 was fused to two ligand-binding domains, one derived from a calmodulin-binding peptide (CBP), the other from protein A. This arrangement endows the bait protein with high affinity for both calmodulin and IgG affinity resins. The calmodulin-binding peptide and protein A affinity tags are separated by the recognition sequence for tobacco etch virus (TEV) protease, permitting proteolytic elution of the fusion PIAS-CBP protein from the IgG affinity resin. The TAP-method has been used very successfully to isolate protein complexes from cultured yeast cells (Puig et al., 2001; Rigaut et al., 1999). Similarly, successful applications in mammalian cells have also been reported (Gavin et al., 2002; Westermarck et al., 2002). Currently, the group of Prof. Dr. Eilers is establishing the TAP-method at the IMT, in Marburg.

Although there is no doubt that the TAP-method allows the efficient isolation of protein complexes from *S. cerevisia*e, there remain some potential disadvantages. For instance, the epitope-tags used, especially the protein A-epitope, is rather big and may interfere with protein interaction surfaces in a protein complex. In addition, IgGs present in high amounts in serum might interfere with the protein A tag.

In order to avoid these problems we decided to use the Biotin-avidin system.

3.4.3.2. The Biotin-Avidin System

Prominent among the affinity-based purification methodologies is the Biotin-Avidin system. Biotin is a naturally occurring cofactor for metabolic enzymes, which is active only when covalently attached to enzymes through the action of specific protein–Biotin ligases. Any biotinylated substrate can be bound very tightly by the proteins Avidin and Streptavidin. Biotin-Avidin binding is the strongest noncovalent interaction known in nature (Kd 10^{-15} M), several orders higher than that of commonly used antibodies or other affinity tags. As a result, the Biotin-Avidin affinity system has numerous applications in modern biological techniques. For the

purposes of protein purification, in particular, biotinylation offers a number of advantages. For example, the high affinity of biotin for Avidin-Avidin allows purification of the biotinylated protein under high stringency conditions, thus reducing background binding often observed with other affinity tags that elute more easily. In addition, there are very few naturally biotinylated proteins, thus reducing the chance for cross-reaction when using biotinylation in protein purification, as opposed to antibodies that may cross-react with several species. The advantages of biotinylation tagging has led to the development of sequence tags that are biotinylated in bacterial, yeast, insect, and mammalian cells. These tags, however, are large in size (at least 63 aa) and may thus affect the structure of the proteins they are fused to. Furthermore, biotinylation using these tags is not very efficient, particularly in mammalian cells. Another approach, so far only demonstrated in bacteria, utilizes small (23-aa) artificial tags that have been selected through multiple rounds of screening combinatorial peptide libraries for specific biotinylation by BirA biotin ligase. These tags have been shown to be biotinylated *in vitro* with kinetics comparable to those of natural biotin acceptor sequences and thus may serve as excellent substrates for efficient biotinylation in cells with coexpressed biotin ligases. The BiotinTAG (AVITAG) sequence used for construction of our tags was a unique peptide, just 15 residues long, which is recognized by biotin ligase (Schatz, 1993). In the presence of ATP, the ligase specifically attaches biotin to the lysine residue in this sequence (Fig.3.32).

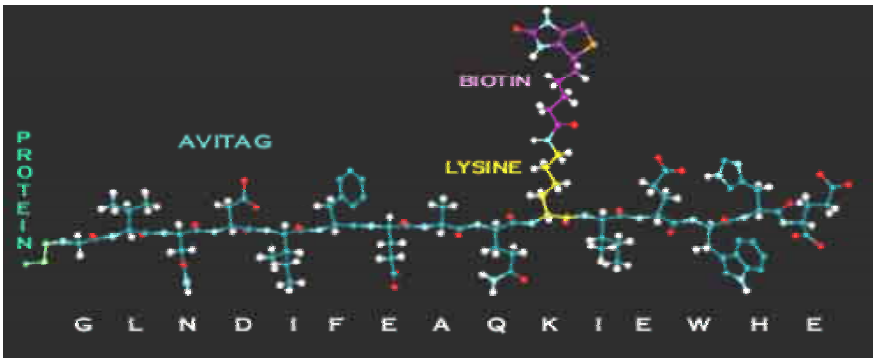


Fig.3.32. The BiotinTAG peptide sequence. Biotin is conjugated to the acceptor lysine highlighted in yellow.

Biotinylation can either occur by the cells endogenous protein–biotin ligases or through the coexpression of an exogenous biotin ligase, in most cases that of the bacterial BirA enzyme. This biotin-protein ligase activates biotin to form biotinyl 5' adenylate and transfers the biotin to biotin-accepting proteins. It also functions as a biotin operon repressor. The protein is encoded by the BirA gene.

We used for our experiments the pBUD-BirA (expression construct described in DeBoer et al., 2003, kindly provided by Harald Braun).

3.4.3.3. The FLAG - BiotinTAG System

The second tag chosen for our tandem affinity purification system was the FLAG tag. Recently, a 3XFLAG (triple FLAG) tag was developed, which is 10-20 times more sensitive than a single FLAG and up to 200 times more sensitive than any other tags (6xHIS, HA, GST, c-myc). Another advantage of the FLAG tags is the presence of an Enterokinase cleavage site (AspAspAspAspLys↓) at the end of the sequence (for details, see FLAG® system homepage at www.Sigma-Aldrich.com).

The tandem affinity purification system based on BiotinTAG and FLAG design for PIAS1 and Sp3 our "proteins of interest" is schematically drawn in Fig.3.33.

PIAS1 and Sp3 were cloned 5' upstream to the triple (3X) FLAG – BiotinTAG. In the first step, the BiotinTAG is biotinylated by exogenous Bir A enzyme expressed upon cotransfection of BirA expression construct (pBud-BirA). In the second step, the biotinylated protein of interest is bound to Streptavidin beads. Avidin, Streptavidin and NeutrAvidin biotin-binding proteins each bind four biotins per molecule with high affinity and selectivity. We chose to use Streptavidin, a bacterially derived biotin-binding protein, because the dissociation constant between biotin and Streptavidin is reported to be about 30 times higher than the dissociation constant of Avidin (Qureshi et al., 2002).

After intensive washing, most of the contaminants are anticipated to be removed. It is expected that Streptavidin beads bind also endogenous biotinylated proteins.

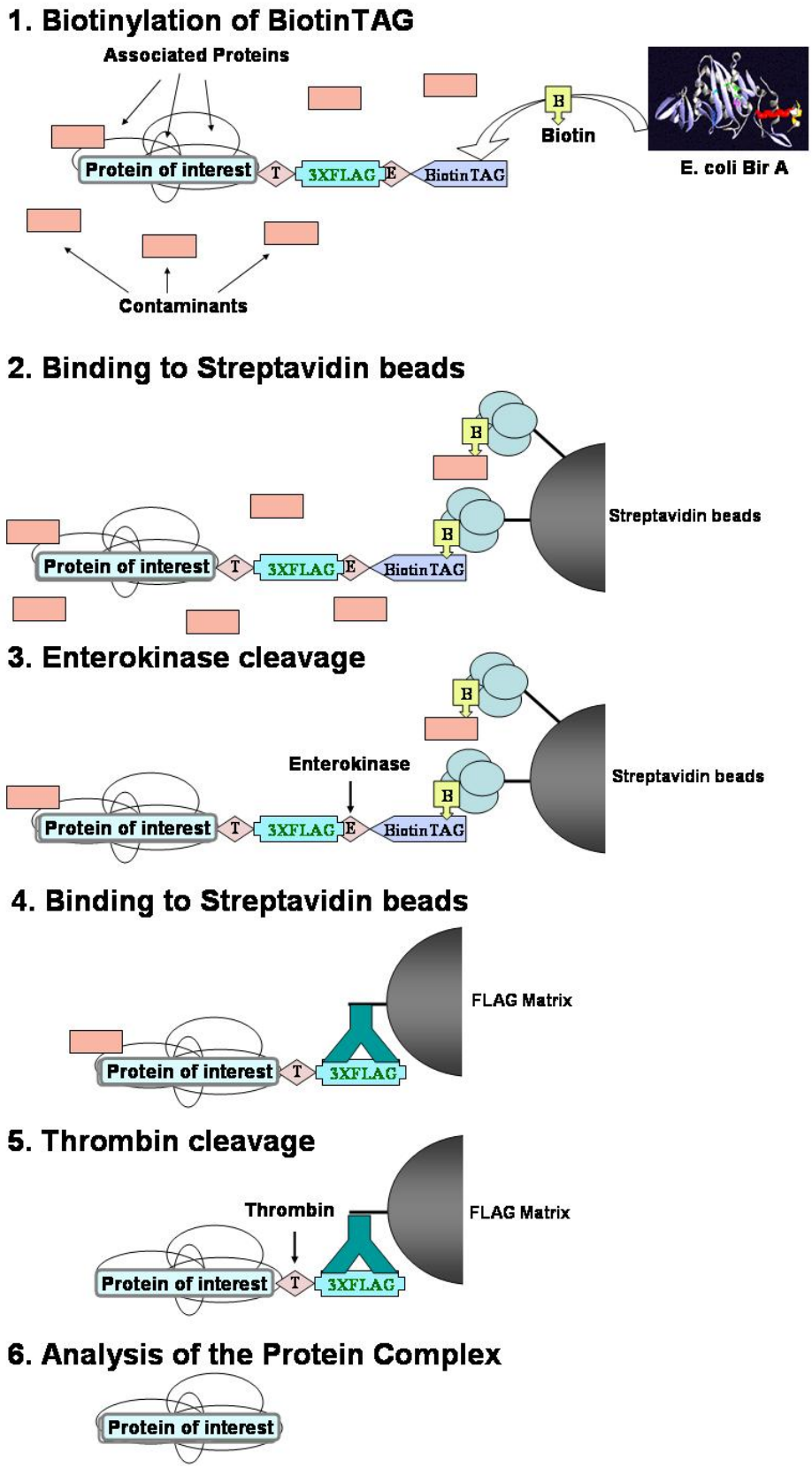


Fig.3.33. BiotinTAG and FLAG based tandem affinity purification system. Explanation: see text. Abbreviations: B - Biotin; T - Thrombin cleavage site; E - Enterokinase cleavage site.

The PIAS1 or Sp3 protein complex is removed from Streptavidin column in the third step by cleaving the BiotinTAG from the fusion protein using site-specific protease Enterokinase, able to recognize its cleavage site that is part of the FLAG tag. In order to get rid of the last contaminants, the protein of interest containing complex is bound in the fourth step to second affinity column. This procedure involves capturing the protein of interest protein with M2 anti FLAG specific antibody directly conjugated to an agarose resin (Sigma). After washing of the protein-FLAG matrix complex, all the remaining contaminants are likely to be washed away.

To elute the FLAG tagged protein of interest from ANTI-FLAG® M2 Agarose Affinity Gel, the addition of the competitor FLAG peptide, glycine and EDTA at pH 3.5 is normally sufficient. To avoid incomplete elution of 3XFLAG tagged PIAS1, a Thrombin cleavage site was cloned between the protein sequence and the tag. The PIAS1 or Sp3 protein complex from the second affinity column in step 5 is further purified in the last step of the purification scheme depicted in Fig.3.33.

3.4.3.4. *In vivo* Biotinylation of Tagged Proteins

In order to verify whether the principle of the protein complex purification strategy was reliable, the functionality of each tag component was tested.

The first question connected to our topic was whether the BiotinTAG is biotinylated by endogenous biotin ligases or by exogenous BirA.

To answer this question the PIAS1 tagged expression construct (pN3-PIAS1FB- see protein structure in schematic drawing of Fig.3.34) was transfected into HEK293 cells independently or with a BirA construct (pBud-BirA). Proteins from cell extracts were separated by 6% SDS polyacrylamide gel electrophoresis. HRP conjugated Streptavidin was used for detection.

As is seen in Fig.3.34, in addition to biotinylated PIAS1, endogenous biotinylated proteins are detected (bands marked with stars -*- in the figure).

In conclusion, the BiotinTAG fused to PIAS1 was biotinylated *in vivo* and the biotinylation occurred only in the presence of overexpressed BirA. Apparently, the

endogenous biotin ligases played no role when PIAS1 FB construct was transfected on its own. It can be seen also that the presence of BirA did not enhance biotinylation of endogenous proteins.

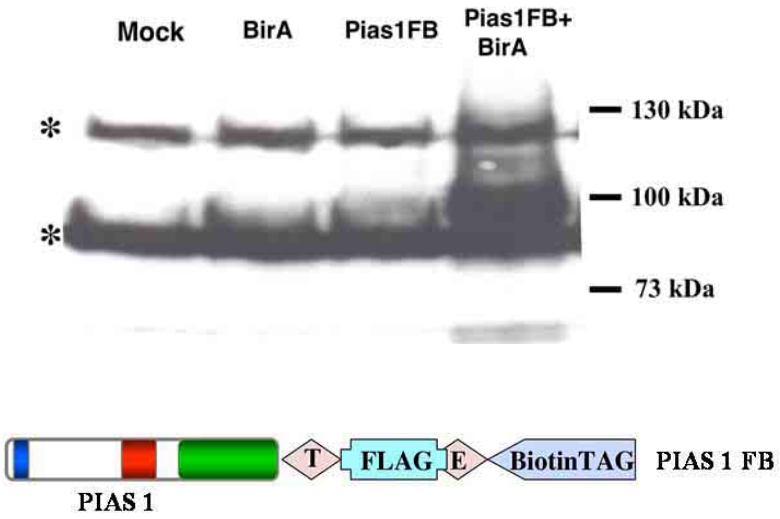


Fig.3.34. Biotin ligase BirA is required for BiotinTAG biotinylation.
HEK293 cells were transfected with expression constructs for PIAS1 (pN3-PIAS1FB) and BirA (pBud-BirA) as indicated. Cells were lysed with SDS-containing buffer. Proteins were separated by 6% SDS polyacrylamide gel electrophoresis. After blotting, the PVDF membrane was incubated with Streptavidin – HRP conjugated. Stars (*) indicate the endogenous biotinylated proteins. Schematic drawing represents PIAS1-FLAG -BiotinTAG fusion protein. Color code for PIAS1: The blue box represent the SAP (SAFA, Acinus, PIAS) domain, the red box indicates the RING-like domain and the green box the Serine-rich region.

Furthermore, the efficiency of binding to Streptavidin beads of biotin- tagged PIAS1 was tested. Cell extracts from HEK293 cells cotransfected with biotin tagged PIAS1 and BirA, were incubated with Streptavidin beads and after incubation, unbound proteins were analyzed. I did not observed detectable PIAS1- biotinylated in flow-through, all biotinylated PIAS is bound to beads in a high efficient manner (data not shown).

In addition to PIAS1, I cloned also Sp3 as fusion to both FLAG and 3XFLAG -BiotinTAG. The main reason was the need to use in parallel, during protein purification protocols, another protein as a control. Two unrelated proteins are

expected to have, at least partially, different protein complex partners. Another reason was the possibility to detect Sp3 in Western Blot after removing the FLAG-BiotinTAG by site-specific proteases because the PIAS1 detection is difficult due by anti PIAS1 antibodies low specificity (see 4.3.1, footnote)

The Sp3 and PIAS1 expression constructs cloned as fusion with FLAG or 3XFLAG- BiotinTAG are depicted in the schematic drawings of the Fig.3.35.A. Both constructs were transfected into HEK293 cells with a BirA expression construct (pBud-BirA) and were detected by Western Blot using Streptavidin – HRP conjugated.

Fig.3.35.B shows that all Sp3 and PIAS1 tagged constructs are expressed, biotinylated and detectable by Western Blot analysis. I also used, successfully, anti-FLAG antibody for Western Blot detection of those constructs (data not shown).

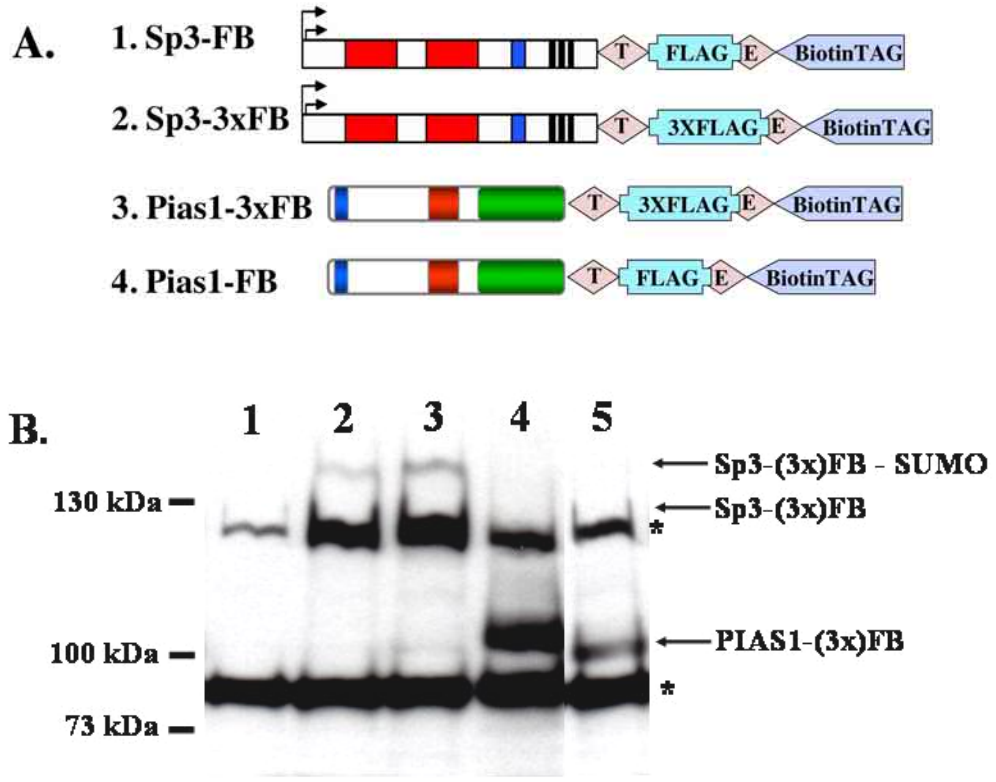


Fig.3.35. Tagged PIAS1 and Sp3 constructs.
A. Schematic drawing of Sp3 and PIAS1 tagged expression constructs.
B. HEK293 cells were transfected with expression constructs for Sp3 (pN3-Sp3FB lane **2**, pN3-Sp3-3XFB lane **3**), PIAS1 (pN3-PIAS1FB lane **4**, pN3-PIAS1-3XFB lane **2**) along with BirA (pBud-BirA). In lane **1** are nontransfected control cells. Cells were lysed with SDS-containing buffer. Proteins were separated by 6% SDS polyacrylamide

gel electrophoresis. After blotting, PVDF membrane was incubated with Streptavidin – HRP conjugated. Stars (*) indicate the endogenous biotinylated proteins.

3.4.3.5. Thrombin and Enterokinase Testing

As mentioned previously, the antibody against PIAS1 was not as sensitive as the anti Sp3 antibody. Therefore, for site-specific protease assessment I used the Sp3 tagged expression construct (Fig.3.36). The Biotin-FLAG tandem tag contains two protease recognition sites, one for Enterokinase (E in the figure) and one for Thrombin (T). The commercially available proteases were provided together with capture buffers in the Enterokinase Cleavage Capture Kit and the Enterokinase Cleavage Capture Kit from Novagen. HEK293 cells were transiently transfected with expression constructs for Sp3 (pN3-Sp3-3XFB) and BirA (pBud-BirA). Cell extracts were incubated with Streptavidin beads. After washing, the beads were incubated with Enterokinase or Thrombin as per manufacturer instructions. Fig.3.36 depicts one of the trial experiments for each protease. The input cell extract (**I**) without any intervention contained endogenous and exogenous tagged Sp3 protein. The control (**C**) was Streptavidin beads incubated with cell extracts **(S)** in the presence of cleavage buffer without Thrombin in order to check if the buffer may influence tagged protein-matrix binding. It is revealed that all biotin tagged Sp3 is bound to the Streptavidin beads and is not detectable in the flow-through (**FT**).

The Streptavidin beads coupled with biotinylated Sp3 were incubated with 10U (5 µl from 2U/ µl stock) of recombinant Thrombin and 10U (5 µl from 2U/ µl stock) of recombinant Enterokinase at RT for incubation times noted in the figure. Sp3 tagged protein bound to Streptavidin beads after incubation with the proteases (**S)** and Sp3 cleaved from beads by Thrombin or Enterokinase detectable in flow-through (**FT**) fractions were analyzed. The site specific proteases Thrombin and Enterokinase did not remove the tags with satisfying efficiency (Fig.3.36). Many causes might impair the proteases enzymatic activity. I tried to solve a part of them by changing incubation times, protease concentration, and cell extract preparation methods but without success.

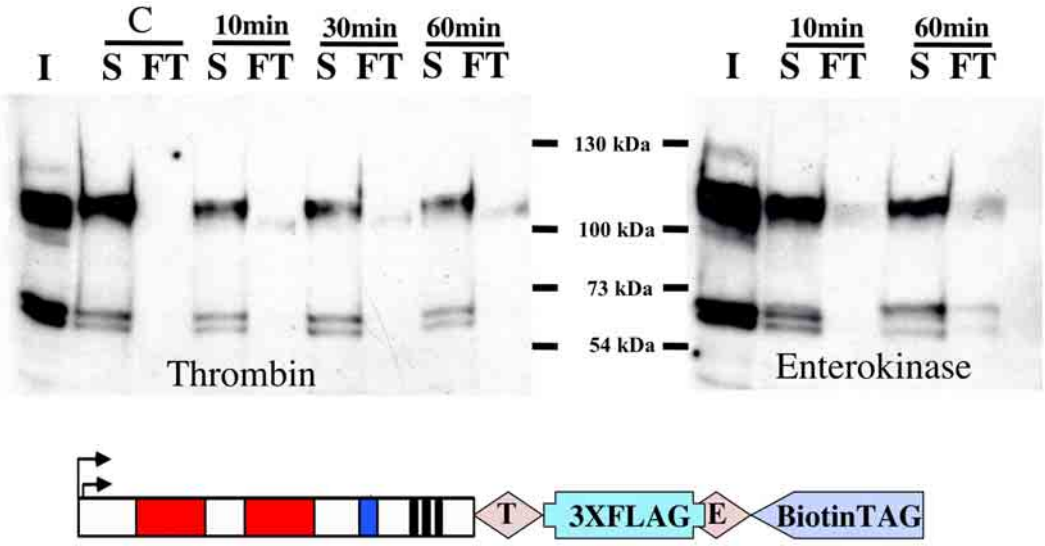


Fig.3.36. Site specific proteases Thrombin and Enterokinase testing.
Sp3–3XFLAG -BiotinTAG and BirA expression constructs were transiently transfected in HEK293. Nuclear extracts were subjected to AP (affinity precipitation) with Streptavidin agarose beads. Biotinylated proteins bound to beads were incubated with Thrombin or Enterokinase. Bound proteins **(S)** and removed proteins **(FT)** were separated on a 6 % SDS-polyacrylamide gel and blotted to PVDF membranes. Detection was by immunoblotting with anti-Sp3 antibody.
Abbreviation: **I** input-NE without AP; **C** control- Streptavidin beads incubated just with thrombin cleavage buffer; **S** Streptavidin beads incubated with nuclear extract; **FT** flow-through after Streptavidin AP. Site Specific Proteases incubation time is indicated above.

3.4.3.6. Calmodulin-TEV-BiotinTAG System

I found that site specific proteases, Thrombin and Enterokinase, did not work in our system. The strategy was changed to utilize a site specific protease already successfully used in the TAP-TAG method, Tobacco Etch Virus (TEV). I replaced the Thrombin recognition site and the FLAG tag with CalBP (Calmodulin Binding Peptide) and a cleavage site for TEV protease by recloning from TAP-TAG expression vector. The most important aspect is that the TEV cleavage site is required to be flanked by spacer sequences for a good functionality. The spacers confer a suitable conformation of the TEV recognition site for it to be easily reached by the enzyme. The BiotinTAG was maintained at the end of the tag.

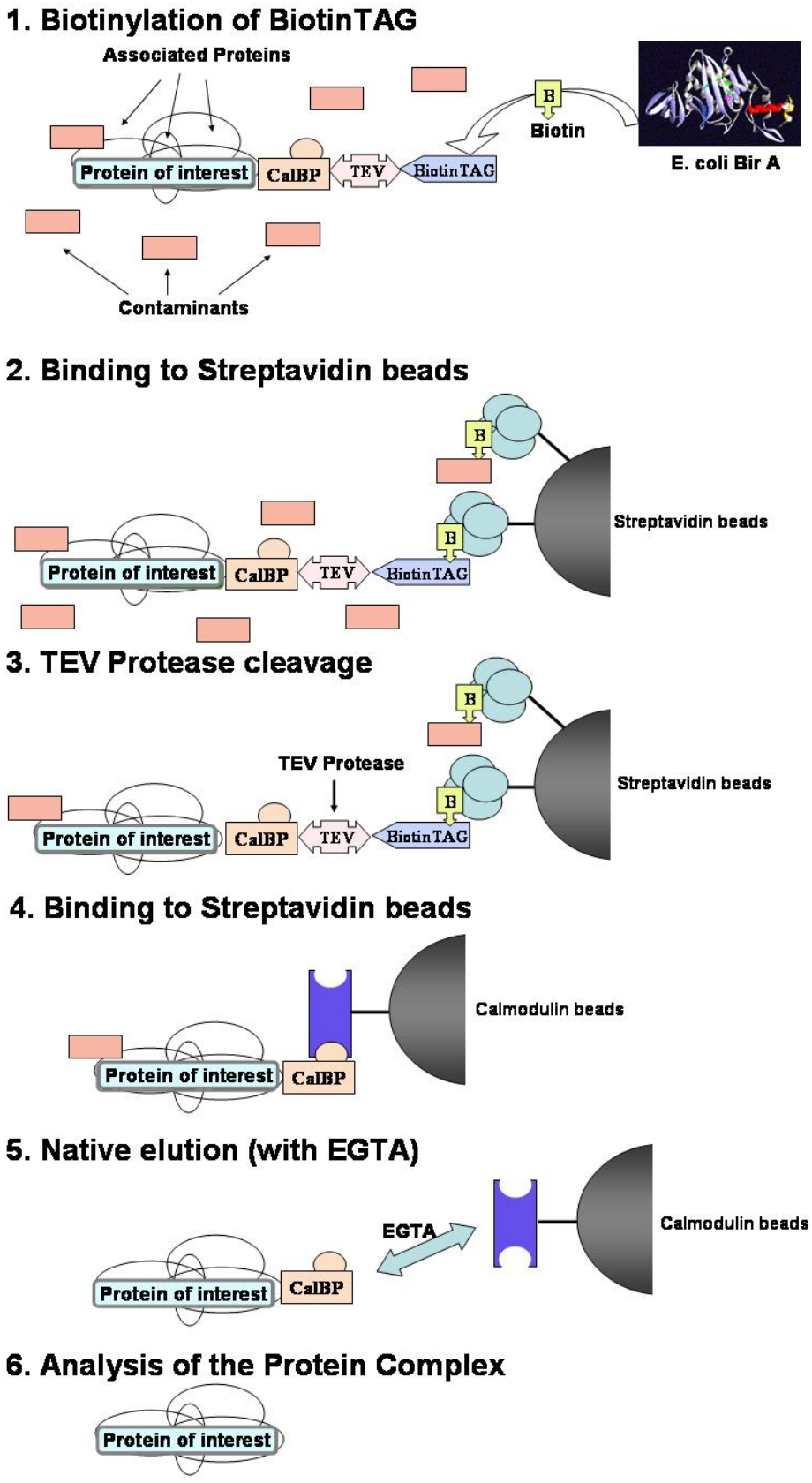


Fig.3.37. CTB tandem affinity purification system. Explanation: see text. Abbreviations: B - Biotin; TEV - Tobacco Etch Virus protease cleavage site; CalBP - Calmodulin Binding Peptide.

The new purification strategy is illustrated in Fig.3.37. Into the MCS of the mammalian expression vector pN3, I cloned full length PIAS1 fused to the tag containing CalBP, TEV cleavage site and BiotinTAG (CTB) resulting in the pN3-PIAS1-CTB expression construct. The first two steps of the strategy are similar to the purification scheme described in Fig.3.33: the tagged protein of interest is biotinylated by exogenous BirA enzyme and then bound to Streptavidin beads. Most of contaminants are removed after intensive washing of the beads. In the third step, the protein of interest containing complex is detached from Streptavidin matrix by TEV protease and is bound, subsequently, to Calmodulin beads in the presence of Ca^{2+} ions.

The remaining contaminants not removed after the first affinity column purification are expected to be washed away at this step. Elution of the protein complex from Calmodulin beads in the next step utilizes Calmodulin Elution Buffer (CEB) containing 0,5M EGTA.

To confirm the efficiency of the new purification method, every part of CTB tag was tested, including binding ability (CalBP and BiotinTAG) and the functionality of TEV cleavage site.

In these experiments, I used the Sp3-CTB tagged construct (Fig. 3.38 schematic drawing) to permit better Sp3 detection by Western Blot following removal of BiotinTAG, using anti Sp3 specific antibody.[2]

HEK293 cells were transfected with pN3-Sp3 –CTB and pBud-BirA expression plasmids. As it can be seen in the Fig. 3.38- input line (I), the Sp3-CTB expression band migrated slower following SDS-PAGE and Western Blot analyses compared to endogenous long isoform bands (liSp3). In the positive control (C+), the Sp3-CTB protein was bound to Streptavidin beads (S) but endogenous Sp3 was found in flow-through (FT).

[2] The CTB tagged protein of interest is detectable with Streptavidin-HRP. After removing of BiotinTAG, an antibody against protein of interest is required. Since PIAS1 detection with anti-PIAS1 antibody was possible at the end of my work due to changing in blocking step (blocking with 1% BSA instead 5% Skimmilk was used), the anti Sp3 antibody for Sp3-CTB construct was used for this experiment.

In order to test the TEV protease cleavage efficiency, HEK293 nuclear extracts containing biotinylated Sp3-CTB tagged protein, were incubated with Streptavidin beads. Most contaminant proteins were removed at this step by intensive washing. The Streptavidin beads were incubated for 2h at 16 ^{0}C with recombinant TEV protease, according to manufacturers recommendations. Following TEV incubation, the flow-through was equilibrated with Calmodulin binding buffer (IPP-150) and subjected to Affinity Precipitation (AP) with Calmodulin beads in the presence of Ca^{2+} for 2h at 4 ^{0}C. The Streptavidin beads (S), Calmodulin beads (Cal) and flow- through (FT) after Calmodulin beads incubation, were analyzed separately in the Fig.3.38 noted as 120 min TEV.

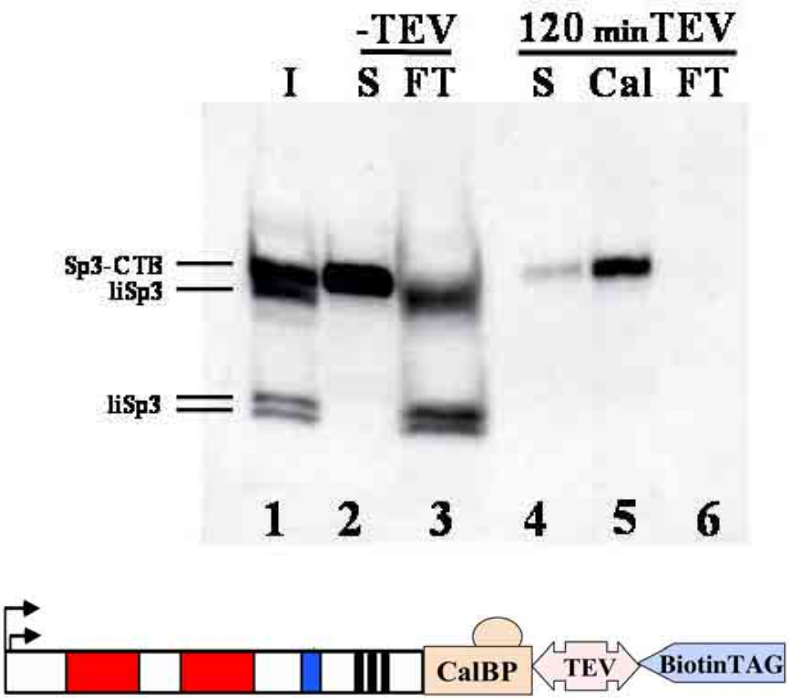


Fig. 3.38 Investigation the cleavage activity of TEV – Tobacco Etch Virus protease.
Sp3–CTB (Sp3-CalBP-TEV-BiotinTAG schematically drawn in lower figure) and BirA expression constructs were transiently transfected in HEK293. Nuclear extracts were subjected to AP using Streptavidin agarose beads. Biotinylated proteins bound to beads were incubated with recombinant TEV and the flow through was affinity purified using Calmodulin beads. Proteins bound to beads or precipitated from flow-through via TCA, were separated by 6.0% SDS-polyacrylamide gel electrophoresis and blotted to PVDF membranes. Detection was by immunoblotting with anti-Sp3 antibody.
Abbreviation: **I** input-NE without AP; **S** - Streptavidin beads incubated with NE from

Sp3-CTB transfected cells; **CAL** Calmodulin beads incubated with FT from Streptavidin beads after TEV incubation; **FT** flow-through after AP. TEV incubation time is indicated above.

Conclusions:

1. The pN3-Sp3-CTB construct was expressed following transient transfection in HEK293 cells. The expression of Sp3 as fusion with CTB tag led to an increase in its molecular weight. This aspect is highlighted in Fig. 3.38 (I-Input line 1) where the Sp3-CTB band was discriminated from endogenous Sp3 long isoforms.

2. Coexpression of pBud-BirA induced efficient biotinylation of the BiotinTAG that allow the binding to Streptavidin beads of Sp3-CTB (Fig. 3.38 –line 2).

3. The TEV protease cleaved the Sp3 tagged construct from the Streptavidin beads (Fig.3.38 - line 4).

4. Exogenous Sp3 with BiotinTAG removed by TEV bound Calmodulin beads (Fig. 3.38 -120 min TEV- **Cal** line5) and was not detected in flow- through (FT) after TCA precipitation (line 6).

3.4.4. Enzymatic Activity of Overexpressed PIAS1

It was revealed in the previous experiments that after overexpression, PIAS1 did not enhance endogenous Sp3 SUMOylation (Fig.3.30) but the bacterially expressed PIAS1 is acting as E3 ligase in vitro (Fig.3.29).

Having established an overexpression system for CTB-tagged proteins, we asked whether overexpressed and affinity purified PIAS1 from mammalian cells can act as an E3 ligase in Sp3 SUMOylation. I tested first the E3 ligase activity of overexpressed PIAS1 coupled to Streptavidin beads without success (data not shown). The question was whether PIAS1 could display the E3 ligase activity after being cleaved from Streptavidin beads by TEV protease.

The same SUMOylation mix consisting of recombinant HA/FLAG-tagged Sp3, recombinant E1 (Aos1 and Uba2 subunits), E2 (Ubc9) enzymes and recombinant SUMO1 was used like for the experiment depicted in Fig.3.29. The bacterially

expressed GST-PIAS1 was incubated with SUMOylation mix (Sapetschnig et al., 2002) as a control for Sp3 SUMOylation (Fig 3.39-line2).

Expression constructs for PIAS1–CTB (pN3-PIAS1-CTB) and BirA (pBud-BirA) were transiently transfected into HEK293. Nuclear extract was subjected to affinity precipitation with Streptavidin agarose beads. Biotinylated PIAS1bound to beads was cleaved from beads by recombinant TEV protease. The free overexpressed PIAS1 was incubated with SUMOylation mix (Fig 3.39-line 4). The SUMOylation mix was incubated with *in vitro* SUMOylation buffer Fig 3.39-line1) and TEV cleavage buffer (Fig 3.39-line3), as controls, in order to check whether the buffers may possibly influence the SUMO modification.

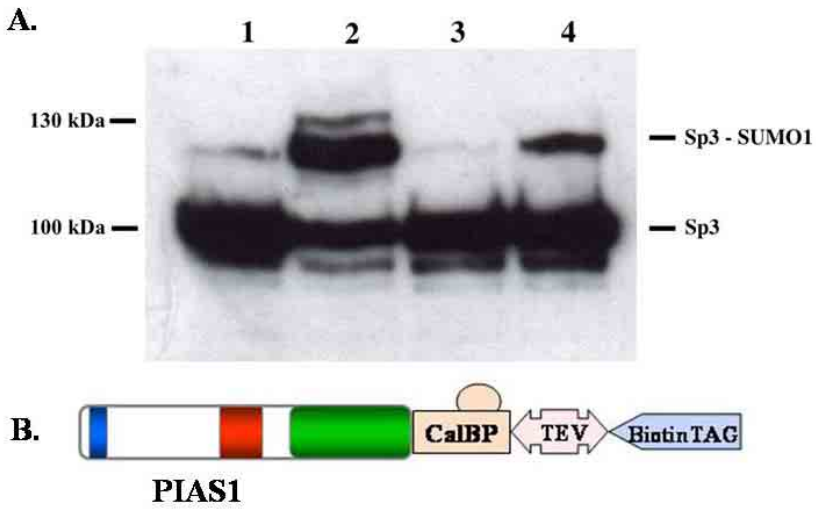


Fig.3.39. Affinity purified PIAS1 from HEK293 cells can stimulate SUMO conjugation to Sp3.

A. In vitro SUMOylation assay.

1. SUMOylation mixture containing HA/FLAG-tagged Sp3 was subjected to SUMO modification in vitro with limiting amounts of recombinant E1 (Aos1 and Uba2 subunits), E2 (Ubc9) enzymes and recombinant SUMO1.

2. SUMOylation mixture incubated with bacterially expressed GST-PIAS1.

3. SUMOylation mixture incubated with TEV cleavage buffer.

4. SUMOylation mixture and overexpressed PIAS1CTB, affinity purified by Streptavidin beads and cleaved from beads with TEV.

Proteins were separated on an 8.0 % SDS-polyacrylamide gel and blotted to PVDF membranes. Detection was performed by immunoblotting with anti-Sp3 antibody.

B. Schematic drawing of PIAS1-CTB. Color code for PIAS1: The blue box represent the SAP (SAFA, Acinus, PIAS) domain, the red box indicates the RING-like domain and the green box the Serine-rich region.

One can conclude from this experiment that after releasing of overexpressed PIAS1 from Streptavidin beads by TEV cleavage, a moderate E3 ligase activity can be observed (Fig 3.39-line4) when compared to the strong enzymatic activity of recombinant PIAS1 (Fig 3.39-line2).

3.4.5. E3 ligases are High Molecular Complexes Components

PIAS-triggered SUMOylation induces the transient recruitment of a transcription factor to subnuclear structures. In these structures a corepressor complex is assembled on the transactivator, leading to inhibition of its activity and gene silencing. As described in detail in the text, an analogous model can explain the context-dependent coactivator role of PIAS proteins. (Schmidt and Müller, 2003)

Hence, raises the critical question: whether PIAS1 is a protein complex member or is just a monomer protein.

To address this question, I used SEC (size exclusion chromatography) of nuclear extracts from HEK293 cells transfected with expression constructs for PIAS1-CTB and Sp3-CTB together with biotin ligase BirA.

In order to avoid unspecific protein complexes accumulation due to the high level of chromatin in the nuclear extracts, I treated the nuclear extracts with Benzonase (Sigma).

Fresh nuclear extracts from HEK293 cells, transfected with CTB tagged expression constructs for PIAS1 and Sp3 along with BirA, were subjected to nuclear fractionation throughout Superose6 column. The reason to use Superose6 was that this column has a broader range of separation than Superdex 200 column.

Each fraction was equilibrated with Calmodulin binding buffer (IPP-150) and subjected to affinity precipitation via Calmodulin beads. The proteins bound to beads were separated through SDS-PAGE gels and detection was performed by Streptavidin-HRP conjugated.

The fractionation profiles of CTB tagged PIAS1 and SP3 are depicted in Fig.3.40. Both exogenous proteins are clearly detected in blots and were found to be parts of

protein complexes. Despite the affinity precipitation with Calmodulin beads, traces of endogenous biotinylated proteins are detectable (marked with stars-*).The input controls nuclear extracts (NE) was not subjected to fractionation. Because the Sp3-CTB control is overexposed, for a better discrimination between endogenous biotinylated proteins and Sp3-CTB, a lower exposed band of input was added.

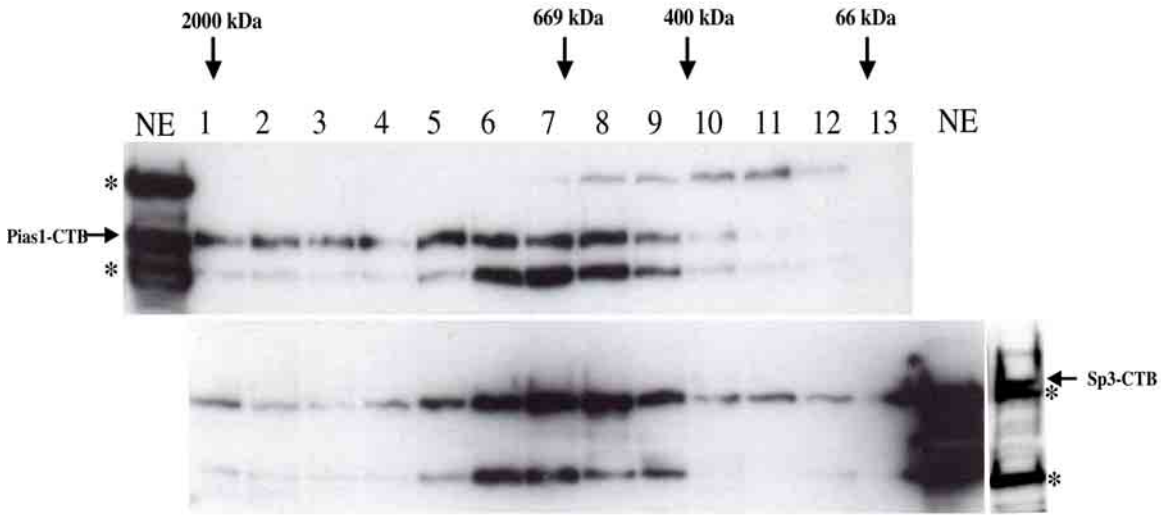


Fig.3.40. Gel fractionation profiles of PIAS1 and Sp3 overexpressed in HEK293 cells.

Expression constructs for PIAS1 (pN3-PIAS1 CTB) and Sp3 (pN3-Sp3 CTB) were transfected in HEK293 cells along with BirA biotin ligase (pBud-BirA). Nuclear extracts were treated with Benzonase and then subjected to size exclusion chromatography by Superose 6 column. Affinity purification of PIAS1 CTB or Sp3 CTB tagged proteins was performed with each fraction using Calmodulin beads via CalBP tag of PIAS1 (upper blot) and Sp3 (lower blot). Proteins bound to Calmodulin beads were separated through 8% SDS polyacrylamide gels. After blotting, PVDF membrane was incubated with Streptavidin – HRP conjugated.
The molecular weigh markers are indicated above the figure. Stars (*) indicates the endogenous biotinylated proteins.

PIAS1 and Sp3 appear to form high molecular complexes. According to observation of another group (S.Philippsen-Rotterdam, unpublished), just PIAS1 can form complexes but not Sp3. To test if these observed complexes are specific or just unspecific accumulation of proteins, we analyzed gel filtration fractions by Western blotting, probing membranes with specific antibodies against different proteins

endogenous proteins Brg1 (A), Tip60 (B), Sp1 (C), and Sp3 (D) in Fig. 4.39.

The first control blot was performed with specific antibody against Brg1, one of the catalytic ATPase subunit of the large SWI/SNF complex protein that is involved to the regulation of gene expression by altering the chromatin structure (Muchardt and Yaniv, 2001). It can be observed in Fig.3.41-A that the Brg 1 is detected at a highest molecular range compared to other proteins analyzed.

Tip60 (Tat interactive protein, 60 kDa), a MYST family HAT first identified as an HIV-Tat interacting protein, is an androgen receptor (AR) co-activator and exerts its effect via directly acetylating the AR, while it counters the deacetylating repressing effect of HDAC1 (Halkidou et al., 2004). This protein (Tip60 B- blot) exhibit an different fractionation profile if compared with Sp1 (C) and Sp3 (D). Another interesting finding is that phosphorylated Sp1 (Fig.3.41-C-upper band), appear to be associated with high molecular protein complexes if compared to unphosphorylated form of Sp1 (Fig.3.41-C-lower band). Also one can observe that Sp3 small isoforms are detected in almost all fractions if compared with Sp3 long isoforms, found predominantly into high molecular weigh fractions Sp3 (D).

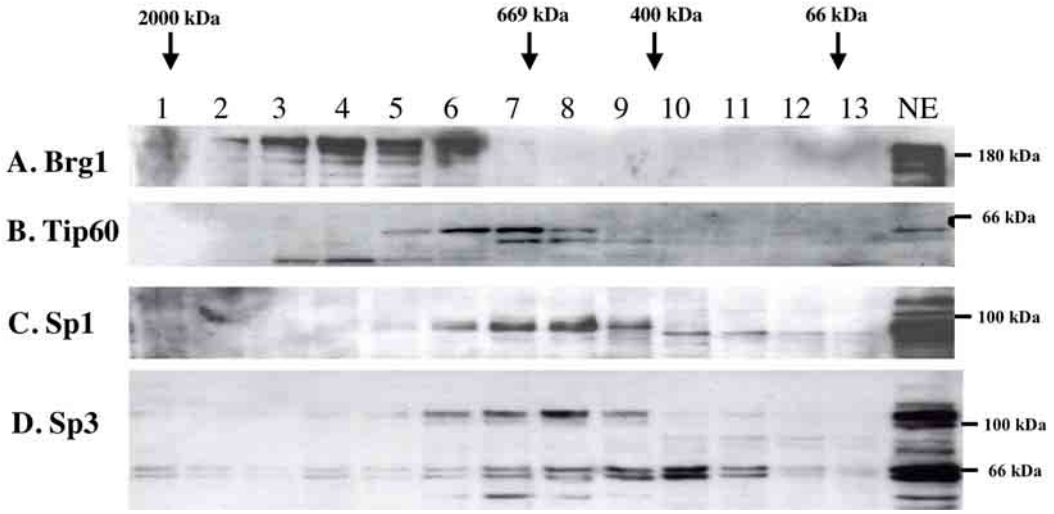


Fig.3.41.Gel fractionation profiles of endogenous Brg1 (A), Tip60 (B), Sp1 (C), Sp3 (D) in HEK293 cells. Nuclear extracts were treated with Benzonase and then subjected to size exclusion chromatography by Superose 6 column and then gel filtration fractions were separated through 8% SDS polyacrylamide gels. After blotting, PVDF membranes were incubated with anti Brg1 SC (A), anti Tip60 SC (B), anti Sp1 serum (C) and antiSp3 SC (D) specific antibodies.

To conclude, the fractionation profiles confirm the association of PIAS1 to protein complex(es). This association appears to be specific because different proteins exhibit specific fractionation patterns.

3.4.6. Tet-Off PIAS1 and Sp3 Expression Systems

Our option for PIAS1 protein complex purification was an inducible stable cell line system, because for purified complex protein analysis high amount of transfected cells is required. Expression of proteins of interest after transient transfection is not the best option because it is expensive and on the other hand, transfected protein is expressed in very high level and may recruit unspecific proteins to the physiological complex.

CalBP-TEV-BiotinTAG tagged versions of PIAS1 and as control, Sp3 were cloned in pTRE2pur, a response plasmid that expresses our genes of interest (PIAS1 or Sp3) in Clontech's Tet-Off Gene Expression Systems and Tet-Off Cell Lines using the Tetracycline controllable expression systems (the "Tet Technology"), schematic represented in Fig.3.42.

In *E. coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn 10 transposon. TetR blocks transcription of these genes by binding to the Tet operator sequences (tetO) in the absence of Tetracycline. TetR and tetO provide the basis of regulation and induction for use in mammalian experimental systems.

The first critical component of the Tet Systems is the regulatory protein, based on TetR. In the Tet-Off System, this 37-kDa protein is a fusion of amino acids 1–207 of TetR and the C-terminal 127 aa of the *Herpes simplex* virus VP16 activation domain. Addition of the VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). The tTA is encoded by the pTet-Off regulator plasmid, which also includes a Neomycin-resistance gene to permit selection of stably transfected cells.

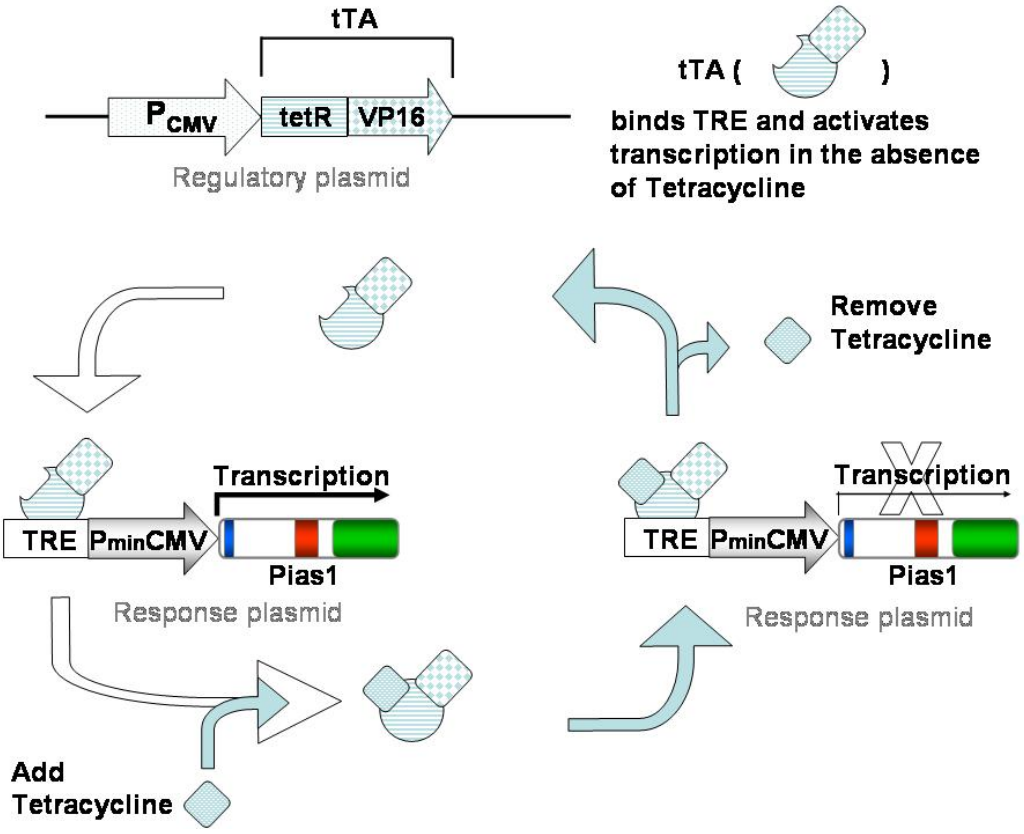


Fig.3.42. Schematic of PIAS1 gene regulation in the Tet-Off System.
The TRE is located upstream of the minimal immediate early promoter of cytomegalovirus (PminCMV), which is silent in the absence of activation. tTA binds the TRE and thereby activates transcription of PIAS1in the absence of Tetracycline.

The second critical component is the response plasmid which expresses a gene of interest (PIAS1 or Sp3) under control of the tetracycline-response element, or TRE. The pTRE contain the TRE, which consists of seven direct repeats of a 42-bp sequence containing the tetO, located just upstream of the minimal CMV promoter (PminCMV). PminCMV lacks the strong enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is low background expression of PIAS1 or Sp3 from the TRE in the absence of binding by the TetR domain.

The ultimate goal in setting up a functional Tet System is creating a double stable Tet cell line, which contains both the regulatory and response plasmids. When cells contain both the regulatory (pTet-Off) and the response (e.g. pTRE- PIAS1-CTB) vectors, PIAS1-CTB is only expressed upon binding of the tTA protein to the TRE and

activates transcription in the absence of Tetracycline in a precise and dose-dependent manner (Fig.3.42).

Given that each cell line has a different sensitivity to G418, Puromycin and Zeocin, titration to determine the optimal concentration of drug for selection was required (see Methods - Kill Curves). For HeLa Tet-OFF cells, I have found 400 µg/ml of G418 to be optimal, the optimal level of Puromycin was typically around 1 µg /ml and for Zeocin the level was 80 µg /ml.

Before transfection with PIAS1 and Sp3 expression constructs, the HeLa Tet-Off cells (stable transfected with the regulatory plasmid), were tested for activity of Tet regulatory plasmid already stable transfected by monitoring of Luciferase intensity after transfecting the pTRE2pur-Luc Control Vector, used as a reporter of induction efficiency (Fig.3.43).

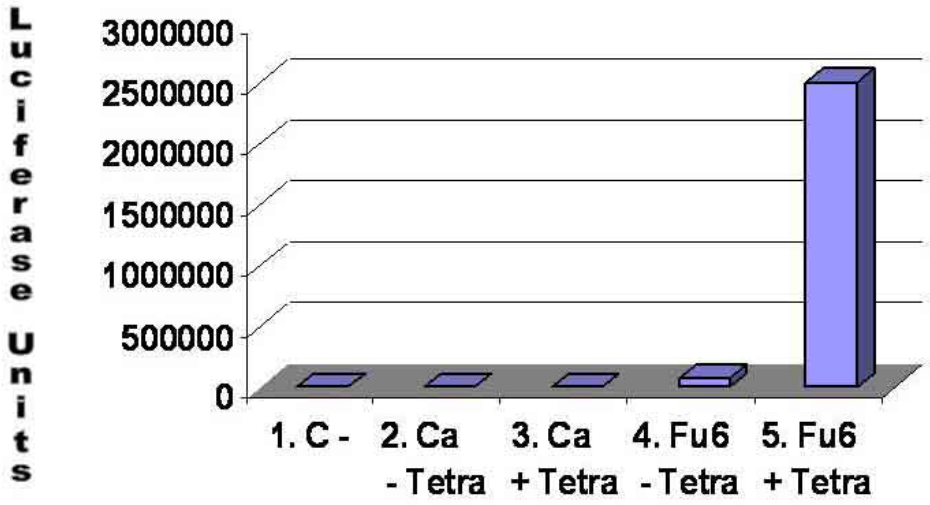


Fig.3.43. Monitoring the induction of Luciferase activity in HeLa Tet-Off Cells when tested by transient transfection with pTRE2pur-Luc. One µg of the pTRE2pur-Luc Control Vector was transfected by calcium phosphate (Ca lines 2 and 3) and Fugene6 (Fu6 lines 4 and 5) transfection methods, in the absence (-Tetra) or in the presence (+Tetra) of Tetracycline. Negative control (C- line 1) represents cells transfected with pTRE2pur vector.

Transient transfections with pTRE2pur-Luc by calcium phosphate and Fugene6 transfection methods were performed, in the presence or absence of Tetracycline. In the cells used for stable transfection experiments, after Tetracycline removing, the induction was significantly high.

After stable transfection into HeLa Tet-OFF of the pTRE2purPIAS1CTB or Sp3CTB constructs (schematically drown in Fig.3.44), selection was performed with Puromycin (see Methods). Simultaneously, the selection antibiotic (G-418) for the Tet-Off regulatory plasmid was added into the medium. Biotin ligase BirA expression constructs (under tetracycline control pBUD-tTA-BirA or normal pBUD-BirA) were transfected (Zeocin selection) together with PIAS1 and Sp3 or following pre-selection with Puromycin.

The next step screened for induction and expression of stable transfected PIAS1 and Sp3 clones. For PIAS1 I tested fifteen clones, eight double transfected with tetracycline controlled BirA and seven double transfected with unregulated (pBUD-BirA). As well, ten Sp3 clones were analyzed, five cotransfected with tetracycline controlled BirA and five colonies cotransfected with pBUD-BirA.

In Fig.3.44-A the screening for expression of one stable cotransfected pTRE2pur-PIAS1-CTB with pBUD-BirA (the colony nr.5) is depicted. Fig.3.44-B and C displays the expression screening of one colony (nr.13) of stable transfected pTRE2pur-Sp3-CTB and pBUD-BirA.

As controls, the same expression constructs (schematically drawn in Fig. 3.44), used for making stable transfected PIAS1 and Sp3 cell lines, were transiently transfected in HEK293 cells together with Tet-Off regulatory plasmid (pTet-OFF) and Biotin ligase BirA (pBUD-tTA-BirA).

In PIAS1-CTB stable transfected clones there was not inducible expression detectable by Western Blot with Streptavidin-HRP. In the example presented in Fig.3.43-A, the expression of pTRE2pur-PIAS1-CTB construct was clearly detectable after transient transfection with BirA into HEK293 cells (positive control - C+) but not in a stable transfected clone. The weak band present in the range of PIAS1-CTB expression was an endogenous biotinylated protein.

Interestingly, the stable Sp3-CTB exhibited a specific band in Western Blot following detection using anti Sp3SC antibody (Fig.3.43-C), which was clearly separated from endogenous Sp3 long isoform (liSp3) or endogenous biotinylated proteins (Fig.3.43-B). The expression of tagged Sp3 in cells treated with Tetracycline was similar to the level seen in induced cells. This observation led us to the conclusion that Sp3-CTB expression in Tet-OFF cells appeared not to be Tetracycline controllable.

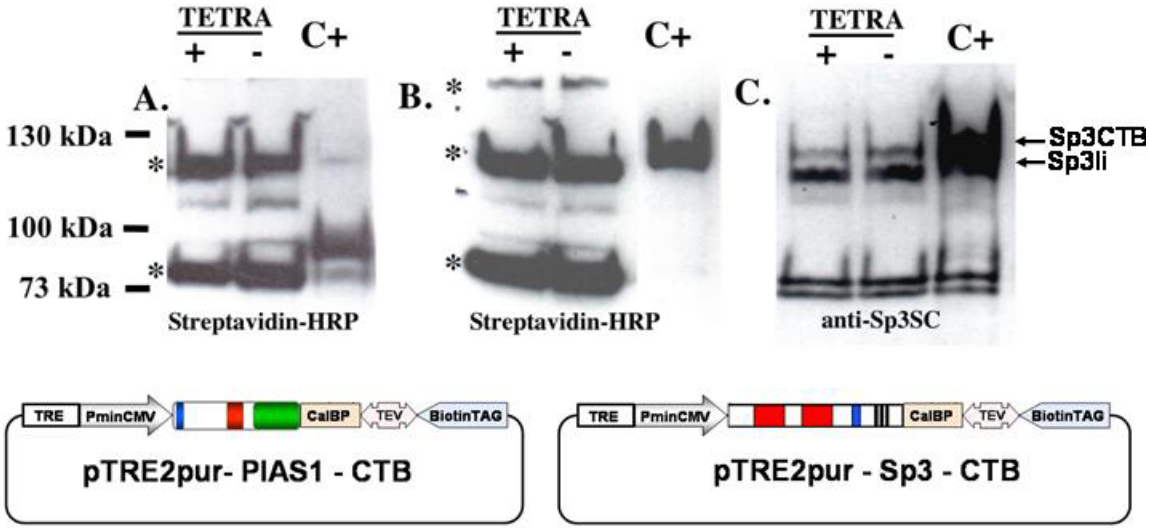


Fig.3.44. Examples of stable transfected PIAS1 and Sp3 clones in HeLa Tet-OFF cells.

A. Stable transfected HeLa Tet-Off cells with expression constructs for PIAS1 (pTRE2pur-PIAS1-CTB) and Biotin ligase BirA (pBUD-BirA) in the presence or absence of Tetracycline. C+ represents transient transfected HEK293 with Tet-Off regulatory plasmid (pTet-OFF), Biotin ligase BirA (pBUD-tTA-BirA) and PIAS1 (pTRE2pur-PIAS1-CTB).

B. and **C.** Stable transfected HeLa Tet-Off cells with expression constructs for Sp3 (pTRE2pur-Sp3-CTB) and Biotin ligase BirA (pBUD-BirA) in the presence or absence of Tetracycline. C+ represents transient transfected HEK293 with Tet-Off regulatory plasmid (pTet-OFF), Biotin ligase BirA (pBUD-tTA-BirA) and Sp3 (pTRE2pur-Sp3-CTB).

Proteins were separated by 6.0% SDS-polyacrylamide gel electrophoresis and blotted to PVDF membranes. Detection was by immunoblotting with Streptavidin-HRP and anti-Sp3 antibody as indicated.

4. Discussion

4.1 Transcription Factors are Substrates for SUMO Modification

The covalent attachment of the ubiquitin-like SUMO protein to its substrates (SUMOylation) represents, in addition to ubiquitination, one of the best-studied examples of a posttranslational modification that stably joins one protein to another. Although both ubiquitination and SUMOylation have important mechanistic similarities and represent evolutionarily ancient and conserved pathways, their physiological consequences are quite distinct. Ubiquitination is generally, but not always, associated with proteasomal protein degradation. By contrast, SUMOylation affects target protein function by altering the subcellular localization of the protein or by antagonizing other modifications (Seeler and Dejean, 2003).

Since the identification of the first SUMO-modified protein, RanGAP, in 1996 (Matunis et al., 1996), a large number of proteins have been shown to be posttranslationally modified by SUMO and new substrates of SUMO-modification continue to be identified at a rapid pace. The currently known SUMO target proteins fall into multiple categories, such as transcription factors, signal transducers, enzymes or viral proteins. Some of the many proteins now known to be modified by SUMO are shown in Fig.4.1.

Recently, a number of transcriptional activators have been recognised as SUMO targets (Gill, 2003; Verger et al., 2003) including steroid hormone receptors (Kotaja et al., 2002), proteins of the C/EBP family (Kim et al., 2002), p53 (Gostissa et al., 1999; Rodriguez et al., 1999), c-jun (Müller et al., 2000) IkBα (Desterro et al., 1998), p73 (Minty et al., 2000), and LEF-1 (Sachdev et al., 2001).

One of the evidence for SUMO modification of Sp3 *in vivo* comes from overexpression experiments in which gene constructs encoding GFP-Sp3WT or a mutant thereof GFP-Sp3K/R were co-transfected along with expression constructs coding for GFP-SUMO1 or GFP-SUMO2 (see Fig. 3.3. Sp3 is SUMO modified *in vivo*).

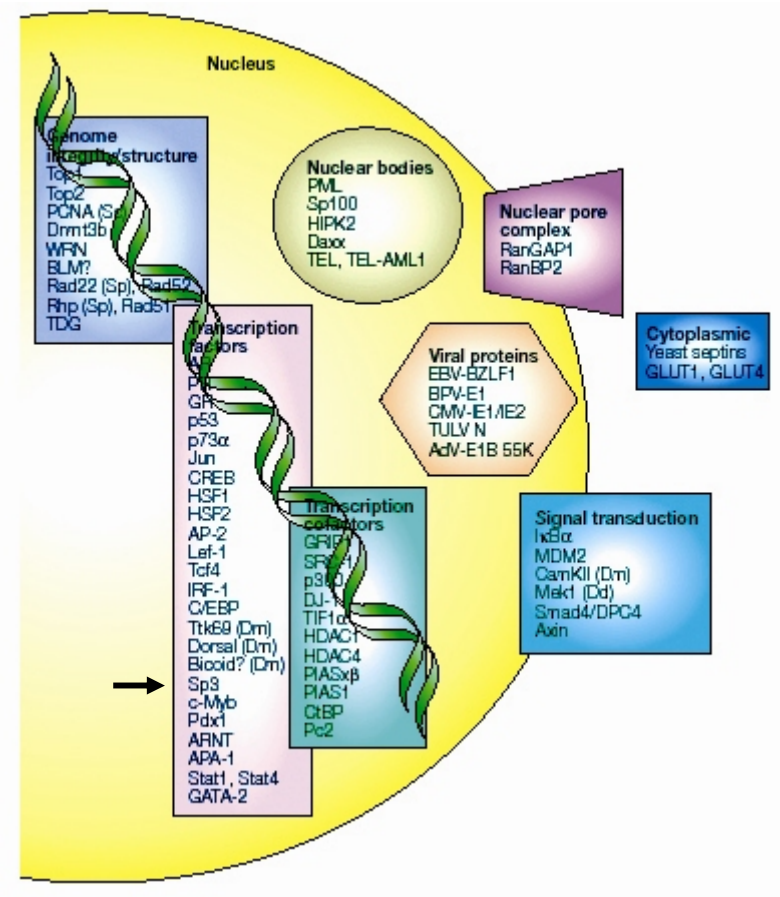


Fig.4.1 SUMO substrates grouped by function.
Budding yeast, fission yeast, *Drosophila* and *Dictyostelium* proteins are indicated by (Sc), (Sp), (Dm) and (Dd), respectively; all others are mammalian proteins.
(Seeler and Dejean, 2003)

A slow-migrating form of GFP-Sp3WT was detected in the presence of SUMO1 or SUMO2 fusion protein demonstrating that both SUMO1 and SUMO2 become efficiently conjugated to Sp3 when overexpressed in cell culture. In contrast, a slow migrating protein was absent with the GFP-Sp3K/R mutant. The slow-migrating band was similarly abundant in the presence of SUMO1 or SUMO2, suggesting that the attachment of both SUMO[3] paralogues to Sp3 occurs with almost equal effectiveness.

The essential questions about the distinctions between different SUMO family members are whether conjugation of particular substrates is restricted to single SUMO

[3] SUMO4 was discovered recently as potential transcript with homologies to the SUMO gene family, after database searches in intron 6 of MAP3K7IP2 gene (Guo et al., 2004). I will refer further just to SUMO1 and SUMO2/3.

paralogue, or whether different paralogues can be used interchangeably. For a few substrates, strong paralogue preferences have been documented, for instance, RanGAP1 is modified almost exclusively by SUMO1 in vivo (Saitoh and Hinchey, 2000). Other substrates, such as the Promyelocytic Leukemia protein (PML), have been reported to be conjugated with both SUMO1 (Sternsdorf et al., 1999) and SUMO2/3 (Kamitani et al., 1998) *in vivo*.

On the other hand, at Sp3 endogenous level, the question of which SUMO paralogue is particularly involved in Sp3 SUMOylation is still open. We observed that different antibodies raised against SUMO1 or SUMO2/3 are not able to recognize in a sensitive manner that would allow detection of SUMO-modified endogenous Sp3 protein, which was affinity purified from mammalian cell extracts.

Also, the issue of specificity is complicated by the fact that paralogue preference can be compromised when individual SUMO proteins are present at superphysiological concentrations (Azuma et al., 2003). Since the conjugation of many proteins to SUMO1 has been demonstrated under conditions where those SUMO proteins are highly overexpressed, categorization of paralogue preference for these substrates cannot be considered definitive until their modification is examined under more physiological conditions. Second, to the extent that there is substrate specificity, it is not known how this specificity arises. This is a particularly interesting question because the same SUMO E1 and E2 enzymes are shared between all paralogues. Finally, it is not known whether the consequences of conjugation to different paralogues are distinct.

Attachment of all SUMOs *in vitro* occurs with almost equal efficiency on the same protein substrates in the presence of only two SUMO-specific enzyme activities known as E1 activating, and E2 conjugating enzymes (Tatham et al., 2001).

4.2. SUMO Consensus Modification Sites of Sp3

The major SUMO acceptor lysine in the transcription factor Sp3 has been mapped to a previously described inhibitory domain of the protein (Dennig et al., 1996). Removal of SUMO from Sp3 by mutation of the acceptor lysine and mutations of other residues in the SUMO consensus site, led to a dramatic increase in the transcriptional activity of Sp3 emphasizing that the integrity of the SUMO consensus motif is critical for SUMOylation (Sapetschnig et al. 2002).

Study of endogenous Sp3 modification by SUMO is complicated by the existence of various Sp3 isoforms. Immunoblot analyses revealed four Sp3 distinct proteins, two slow migrating of more than 100 kDa and two fast migrating species of approximately

72 kDa (see Fig. 3.5. Complexity of endogenous Sp3 protein expression). Seven to eight Sp3 bands appear, when cells were lysed in special conditions, the additional bands represent SUMO modified Sp3 isoforms. All signals are specific for Sp3 since extracts prepared from Sp3-deficient embryonic stem cells did not cause any background. The Sp3 SUMOylation takes place at lysine K551 within inhibitory domain, present in all four isoforms.

The SUMO acceptor sites in several other transcription factors such as the glucocorticoid receptor (GR), Myb, and C/EBP (CAAT/enhancer binding protein) have also been mapped to previously identified inhibitory regions of these proteins, and mutation of the major SUMO acceptor sites in these factors leads to an increase in transcriptional activation (Tian et al., 2002; Le Drean et al., 2002; Bies et al., 2002). It is worth noting that the activity of these SUMO-regulated inhibitory domains is not dependent on their position within the protein and the inhibitory function can be transferred to other transcriptional activators (Dennig et al., 1996; Kim et al., 2002).

Many proteins harbor one or multiple SUMO consensus modification sites (□KXE), but only a fraction of these sites are actually modified. It is likely that the SUMO1 conjugation site on target proteins is located in flexible surface loops or termini, as shown in p53 and as predicted for other SUMO1 target proteins where the conjugation sites have been identified.

Sequence analyses of Sp family members, indicated that Sp1, Sp3 and Sp5 carry sequences reminiscent of consensus sites for SUMOylation. As shown in Fig.3.9 (Other potential SUMOylation sites in Sp3), three lysines embedded within SUMO consensus motifs were noted at amino acids 9 (V<u>K</u>QE), 120 (I<u>K</u>DE) and 551 (I<u>K</u>EE) of Sp3. Putative sites of SUMOylation were also noted at lysine 16 and lysine 391 of Sp1 and Sp5, respectively. So far, there are no reports concerning Sp1 and Sp5 modification by SUMO. Nonetheless, our experiments regarding Sp1 expression in immunoblotting, did not give us any indication about Sp1 SUMOylation.

The well-known example of SUMO target proteins harboring multiple SUMO consensus sites is Topoisomerase I (TOP1). Topoisomerase I is required to remove negative supercoils from DNA (Watts, 2004) and is a target for the anti-cancer drug camptothecin (CPT). Treatment of mammalian cells with CPT results in the appearance of at five SUMO modified forms of TOP1 which differ in size by around 20 kDa (Mao et al., 2000).

Despite the fact that Sp3 also bears three SUMO consensus sites, we never observed three SUMO modified forms of Sp3 in immunoblotting. The controversy

upon additionally potential Sp3 SUMO motifs was elucidated by carefully analyzing expressions of the full-length versions of wild-type Sp3 (containing all three potential SUMO motifs) or the K551R mutant either HA-epitope tagged at the N terminus or the C-terminus (see Fig. 3.10. Posttranslational modification of full-length Sp3 by SUMO). SUMO-modified Sp3, visualized in immunoblot as a single, slower migrating species, was detected with the wild-type Sp3 construct but not with the Sp3K551R construct. This result strongly suggests that exclusively K551 becomes SUMO-modified but not K9, K120 or any other lysine residue of the Sp3 sequence.

Nonetheless, it was reported that Sp3 may have, beside K551, an additional SUMO motif (K120) which undergoes SUMOylation (Ross et al., 2002). Western blot analysis of extracts from cells transfected with wild-type Gal4-Sp3 or constructs containing to arginine mutations corresponding to position 120 and 551 of full length Sp3, showed that there was one major SUMOylated form of wild-type Gal4-Sp3 as well as at least one minor form. Mutation of K551 completely abolished the major sumoylated form of Gal4-Sp3. In contrast, mutation of K120 had no effect on the major SUMO1-modified form of Gal4-Sp3 but appeared to disrupt the slower migrating minor SUMOylated form. When both lysines K120 and K551 were mutated to arginine, no SUMO1-modified forms of Gal4-Sp3 were detected. Nevertheless, the poor quality of immunoblots as well as abnormal migration of SUMOylated Sp3 bands led us to conclusion that the assertion of Ross and coworkers regarding SUMOylation of lysine 120 within Sp3 sequence is not convincing at all.

4.3. Competition for Target Lysines

As lysine residues can act as acceptors for a range of protein and non-protein modifiers (e.g. SUMO, ubiquitin, NEDD8, methylation and acetylation) it is entirely plausible that covalent addition of SUMO or other modifiers to particular lysine residues could act as a switch to direct the modified proteins into complexes with different functions.

The alternative modification by SUMO or ubiquitin at the same lysine residue has been described for PCNA (proliferating cell nuclear antigen) protein (Hoege et al., 2002). PCNA acts as a ‘sliding clamp’ on DNA, and is involved in several different processes associated with replication, including leading and lagging strand DNA synthesis, mismatch repair, nucleotide- and base-excision repair, post-replicative error-prone and error-free repair and cell cycle arrest. Ubiquitination of PCNA,

however, is not implicated in protein degradation, but plays a pivotal role in DNA repair. Intriguingly, SUMOylation seems to inhibit the role of PCNA in DNA repair. This led to the idea that lysine residues that are affected by alternative modifications may function as regulatory switchboards that can direct proteins to alternate functions (Hoege et al., 2002). The switch of modifications may allow the exchange of binding partners and thus control protein-protein interactions. However, biochemical analysis of the modification status of PCNA in yeast indicates that only a small proportion of PCNA contains covalently attached SUMO or ubiquitin (Hoege et al., 2002).

The role of SUMO in protein stabilization has most convincingly been illustrated on the inhibitor of nuclear factor kappa B alpha (IkBα). In this particular case SUMO modification occurs at the same lysine residue that is used for ubiquitination (Kagey et al., 2003). Whereas ubiquitination of Lys-21 in IkBα requires phosphorylation of the adjacent Ser-32 and Ser-36 residues, SUMO1 preferentially targets the stable, unphosphorylated form of IkBα, indicating that it acts antagonistically to ubiquitin to protect IkBα from degradation (Desterro et al., 1998).

Another example of antagonism of posttranslational modifications to a particular lysine is regarding Sp3 transcription factor. Originally, it was demonstrated that Sp3 is an acetylated protein in vivo. Strikingly, a mutant of Sp3 that lacks the inhibitory domain containing the lysine residue of the IKEE sequence exhibited a far weaker acetylation compared to wild type (Braun et al., 2001). This result suggested originally that acetylation of this lysine is responsible for silencing.

Nevertheless, the experiments described later on (Sapetschnig et al., 2002) which identify the lysine of the IKEE sequence as a substrate for SUMO modification, led to reconsideration of the role of acetylation. At that time, the experimental data suggested that the same lysine residue can be modified by either acetylation or SUMOylation. Since both covalent modifications occur at the ε-amino group of the lysine, acetylation would prevent SUMO modification and vice versa. SUMO modification silences Sp3 activity since all mutations that prevent SUMOylation relieve inhibition. However, recent experiments indicate that antibodies highly specific for the acetylated IKEE motif of Sp3 did not recognise endogenous Sp3 or Sp3 over-expressed in 293 cells or SL2 cells, suggesting that lysine residue K551 is not acetylated *in vivo* or the acetylation level of this particularly lysine is under detection limit of the antibodies. Most likely, the acetylation happens at other lysine residues in the DNA-binding domain.

Other Sp/KLF factors are also acetylated in the zinc finger DNA-binding domain (Suzuki et al., 2003). EKLF/KLF1 is acetylated by p300 and its homologue CBP at two lysine residues, one residing in the DNA-binding zinc finger domain and the other in the transactivation domain. The mutation of the zinc finger acetylated residue does not affect DNA binding activity and the individual role of its acetylation is unclear, but mutation of the transactivation domain lysine residue results in decreased transactivation and acetylation collectively increased affinity for the SWI/SNF chromatin remodeling factors (Zhang and Bieker, 1998; Zhang et al, 2001).

4.4. SUMOylation and Subcellular Localization

Depending on the target protein, SUMOylation can occur in the cytoplasm or nucleus, and this modification could be involved in regulating the subcellular localization of a number of substrate proteins (Hilgarth et al., 2004).

Posttranslational modification by SUMO has been shown to regulate subcellular localization of many targets including RanGAP, the first identified SUMO substrate (Matunis et al., 1996). RanGAP is a small GTP transactivating protein that plays a role in nuclear import. Unmodified RanGAP is cytoplasmic, whereas SUMO modified RanGAP is associated with the nuclear pore (Matunis et al., 1996; Mahajan et al., 1997). SUMO modification of RanGAP greatly increases its interaction with RanBP2 (also known as Nup358), a component of the nuclear pore complex and a SUMO E3 ligase (Pichler et al., 2002). Localization of the RanBP2 SUMO E3 ligase at the nuclear pore may contribute to a broad role for SUMO in regulation of nuclear trafficking (Melchior et al., 2003). In mammalian cells, SUMO modification of several substrates has been linked to nuclear import. In the case of the IkB kinase regulator NEMO, for example, fusion of NEMO to SUMO was sufficient for localization to the nucleus (Huang et al., 2003).

As well as controlling protein subcellular targeting, SUMO has a role in controlling gross subnuclear architecture. This is best exemplified by the PML NBs, the integrity of which is dependent on the SUMOylation of their principal component, PML.

The promyelocytic leukemia (PML) nuclear bodies (NBs), also known as ND10 or PODs (PML oncogenic domains), represent one of the best-studied examples of a subnuclear structure, the integrity of which is altered in human disease. PML was first discovered as part of the PML–RAR-α oncogenic fusion protein with the retinoic acid receptor-α (RAR-α) in cells from patients with acute promyelocytic leukemia (APL), in which expression of PML–RAR-α provoked the striking desegregations of the PML

NBs (Seeler and Dejean, 1999).

Visualization of PML body dynamics in living cells, lead to define three classes of PML bodies, based on their mobility (Muratani et al., 2002).

Stationary PML bodies, and those that exhibited limited localized movement, are indicative of structures that are stably associated with an underlying nuclear structure or with a specific nucleic acid or protein complex, and therefore may represent a mechanism of epigenetic control of gene expression. For example, several studies have previously shown an association between PML bodies and centromeres or other heterochromatin-enriched regions (Seeler et al., 1998). Alternatively, these bodies may be involved in transcriptional regulation by modulating local pools of soluble factors that may be essential for transcription. A variety of such proteins (Sp100, PML, Daxx, pRB and p53) have been reported to localize at least partially to PML bodies (Zhong et al., 2000).

The PML dots (PODs) have also been described also as ‘‘proteolysis centers’’ or ‘‘aggresomes’’ to which misfolded proteins are recruited for degradation under normal conditions. Expression of a mutated form of influenza nucleoprotein that was misfolded and rapidly degraded by proteasomes led to the accumulation of the protein in the PODs upon inhibition of proteasome activity. This was followed by the attraction of proteasomes and poly-ubiquitin to the same structures, suggesting that the PODs may function as a site for proteasomal degradation of ubiquitinated proteins (Misteli et al., 2001).

The most intriguing class of PML bodies was revealed to exhibit metabolic energy-dependent dynamics within the cell nucleus. This group of nuclear bodies, although small at any given time, may act as nuclear ‘sensors’, traveling within nuclear regions and associating with populations of foreign or ‘suspect’ proteins, including viral proteins. The previously observed initial association between PML bodies and nuclear sites of viral DNA transcription and replication, and their subsequent break-up, as well as the increased number of PML bodies present after interferon treatment, is consistent with the possibility that PML bodies act as ‘nuclear sensors’ and could constitute a nuclear defense mechanism. Alternatively, this group of bodies may be involved in associating with or marking misfolded or aggregated proteins within the nucleus (Muratani et al., 2002).

However, Sp3 transcription factor emerge as one of exceptions of SUMO-targets association with PML bodies. Visualization of endogenous Sp3 by immumofluorescence showed a sponge-like, diffuse appearance along with closely

related Sp family members Sp1 and Sp2 that are also located into the nucleus and the subcellular localization patterns are similar to Sp3. Moreover, overexpressed GFP-Sp3 displays a similar localization pattern as endogenous Sp3 protein. Also, the wild-type Sp3 isoforms and the SUMOylation-deficient mutants were located in the nucleus exhibiting the same sponge-like, diffuse appearance. Indubitable, the differences in the activation capacity of the various isoforms and mutants are not due to differences in their subcellular or subnuclear localization.

As well, we found that endogenous SUMO1 shows a different nuclear pattern than Sp3. SUMO1 exhibits a diffuse nuclear distribution but in addition appeared do be accumulated at the rim of the nucleus and in few nuclear dots and emerged not to interfere with Sp3.

Ectopic expression of GFP-SUMO1 led to the accumulation of this fusion protein within PODs, whereas endogenous or overexpressed Sp3 remained diffusely distributed throughout nuclei (see Results 2. Subcellular localization of Sp3). The evident conclusion of these experiments was that the Sp3 was diffusely distributed throughout nuclei and did not accumulate within subnuclear "dots" or PODs after SUMO overexpression or Ubc9 coexpression.

The metabolic energy-dependent nuclear bodies involved in so called "defense mechanism" previously mentioned (Muratani et al., 2002) might enlighten the tough regulation of Sp3 SUMOylation level. One can speculate that SUMO is traveling (as small energy-dependent nuclear body) within nucleus and associate quickly with any "suspect" Sp3 (overexpressed or damaged and misfolded upon stress treatment) leading to rapid degradation of the "suspects" and keeping the Sp3 SUMOylation level constant.

Very quick interaction with energy-dependent nuclear bodies and also difference in size compared with stationary PML bodies may explain why we did not find any redistribution of Sp3 upon SUMO1 overexpression.

In contrast to our results, another group (Ross et al., 2002) utilized anti-Sp3 antibodies and indirect immunofluorescence to detect endogenous Sp3 proteins in mouse P19 embryonal carcinoma cells. These studies localized Sp3 to the nuclear periphery as well as PODs depending on the method of fixation employed. The ectopic expression of a GAL4-Sp3 fusion protein in P19 cells faithfully reproduced these localization results, whereas a mutated construct lacking lysine 551 localized diffusely within the nucleoplasm. Finally, co-expression of a SUMO1 protease with Gal4-Sp3 mobilized Gal4-Sp3 to the nucleoplasm. The same effect was observed when the

SUMO1 was fused of to the amino-terminus of a lysine 551-deficient Sp3 (Ross et al., 2002).

Nevertheless, we found that in several mammalian cells used for our experiments, the Sp3 protein is distributed throughout nucleus and this distribution is not uniform, but rather with randomic holes in a "sponge-like structure". Most possible, the Sp3 localization to the nuclear periphery observed by Ross and coworkers was a peculiar artifact due to lack of experience in immunofluorescence technique (see Fig. 3.11. Sp3 localization under different permeabilization conditions). Moreover, this bizarre Sp3 localization was never confirmed by another reference.

Regarding Sp3 relocalization in NBs upon SUMO overexpression, the accurate design of the immunofluorescence experiments is crucial for obtaining undoubted results (see Fig.3.20. Sp3 and dot-like structures in Ishikawa cells). In this experiment, Sp3 was forcibly recruited to dots. However, this abnormal recruitment is most likely a forgery produced by fluorophore excitation-emission interference.

One can deduce that many of the experiments exhibiting the relocalization of different SUMO targets to the PODs by employing indirect immunofluorescence methods should be reconsidered. New and accurate microscopy methods which are now available also for investigation of protein-protein interactions (Shav-Tal et al, 2004) hopefully will edify the relocalization of SUMOylated targets controversy.

4.5. Regulation of SUMO Modification at Ligation Step of the SUMOylation Cascade

The initial doubts as to the existence of SUMO E3 ligases were put to rest with the demonstration that members of the SIZ/PIAS protein family fulfill the defining criteria of such factors. First, they have the capacity to interact with both Ubc9 (the E2 enzyme) and the substrate and, second, they can increase the rate of substrate modification. It is considered that E3 ligases likely arose later, during the evolution, achieving the task of substrate discrimination (Hochstrasser, 2000).

Members of the PIAS protein family (PIAS1, PIAS3, PIASx und PIASy) can act as SUMO-specific E3 ligases for certain substrates. Possibly, PIAS proteins exert also functions that are independent of their E3 ligase activity. PIAS1 and PIAS3 were originally described as specific inhibitors of the transcription factors STAT1 and STAT3, respectively, by interfering with DNA binding (Chung et al., 1997; Liu et al., 1998).

PIAS1 was identified in our lab in a two-hybrid screen using the inhibitory domain of Sp3, which contains the SUMOylation motif IKEE, as bait, initially cloned as protein designated SIF-1 (Sp3-interacting protein 1). Sequencing revealed later on that the encoded protein was identical to PIAS1 (Liu et al., 1998). PIAS1 fulfils all requirements to act as an E3 ligase for SUMO conjugation to Sp3. It interacts with the E2 enzyme Ubc9 as well as with Sp3 and SUMO-conjugated Sp3. Most importantly, it strongly stimulates transfer of SUMO1 and SUMO2 to Sp3 *in vitro* (Sapetschnig et al., 2002). It was reported that the nucleoporin RanBP2 that is not related to PIAS proteins also has E3 ligase activity. RanBP2 interacts with the E2 enzyme Ubc9 and enhance SUMO transfer to Sp100, a component of PML nuclear bodies (Pichler et al., 2002). It is worth mentioning that RanBP2 does not act as an E3 ligase for SUMO conjugation of Sp3 (G.Suske and F.Melchior, unpublished data).

Thus far, overexpression of PIAS1 protein in mammalian cells does not enhance endogenous level of Sp3 SUMOylation (see Results- Fig.3.30. Overexpression of PIAS1 does not influence the SUMOylation level of Sp3). The contradiction between the *in vitro* results where PIAS1 strongly enhanced Sp3 SUMOylation and the absence of this effect upon overexpression in cell lines, directs to the idea that the function and specificities of PIAS1 might be regulated by PIAS1-associated proteins.

Recent analyses revealed that PIASxβ, but not PIAS3 and PIASy, could also act as an E3 ligase towards Sp3 *in vitro* (Diplomarbeit B. Stielow). Nevertheless, PIAS proteins enclose an important function in specifying the substrate for modification, although whether, for example, a given PIAS protein can be matched to specific substrates under physiological conditions *in vivo* remains unclear. More likely, additional proteins that control substrate specificity could be involved, as is also the case for ubiquitin E3 ligase complexes (Seeler et al, 2003).

Following analysis of gel fractionation profiles of tagged PIAS1 (Fig.3.40. Gel fractionation profiles of PIAS1 and Sp3 overexpressed in HEK293 cells), we observe that PIAS1 is part of a protein complex(es) within the cell, being detected in a high molecular protein fraction. The identification and subsequent characterisation of the proteins that are part of the observed PIAS1 protein complex will probably unravel partially the SUMOylation mechanism.

Beside the specific SUMO ligase undertakings, PIAS proteins have been found to control the activity of a variety of transcriptional regulators in SUMO independent manner (Schmidt and Mueller, 2002). Transcriptional repression is in many cases mediated by corepressor complexes that contain histone deacetylase activity. The

finding that PIAS proteins can directly interact with histone deacetylase (HDACs) provides further evidence for a role of PIAS proteins in the assembly of such a complex (Tussie-Luna et al., 2002).

Accumulating evidence indicates that the PIAS/SUMO system is involved in targeting to specific subnuclear sites of SUMO-target proteins. PIASy has been shown to inactivate LEF1 possible by inducing its compartmentalization into PML NBs (Sachdev et al., 2001). Considering that NBs are unlikely active sites of transcription, the most simple interpretation of these data is that recruitment to NBs via PIAS/SUMO sequesters LEF1 away from its target genes, thus neutralizing its transcriptional activity. Examination of a tagged LEF1 protein showed localization throughout the nucleus in transfected mammalian cells. Coexpression of PIASy strongly redistributed LEF1 to nuclear bodies, an effect enhanced by coexpression of SUMO1 or SUMO-2. To test whether this effect required the SUMO ligase activity, the authors also transfected the RING mutant of PIASy. Not only did this RING mutant fail to target to nuclear bodies, but it was also unable to redistribute a mutant of LEF1, suggesting that the RING finger and likely the SUMOylation activity of PIASy are important for both the localization of both PIASy and its targets to nuclear bodies. Moreover, this targeting strongly correlated with transcriptional repression by PIASy (Sachdev et al., 2001).

An obvious question is what determines whether the PIAS/SUMO system ultimately leads to the formation of a coactivator or corepressor complex on a given transcription factor. One possibility might be that distinct PIAS forms function as targeting factors to specific subpopulations of NBs, which may contain either corepressors or coactivators. This idea is supported by the observation that at least upon overexpression distinct PIAS forms show a different pattern of subnuclear localization. Hence, PIAS1 exhibits microgranular localization in small foci, whereas PIAS3 concentrates in fewer, but larger granules (Kotaja et al., 2002).

However, my results did not support such observations. I have shown that the Sp3 subcellular localization in HEK293 cells is not affected upon SUMO1/PIAS1 coexpression (FIG.3.28. Subcellular localization of PIAS1 in HEK293 cells). Moreover, the observation that distinct PIAS forms show a different pattern of subnuclear localization could be inaccurate. I observed that upon SUMO1/PIAS1 overexpression, dots in different cells are variable in number and size, microgranular localization in small foci and also larger granules previously described (Kotaja et al., 2002) could be observed in the same slide (FIG.3.28-C).

4.6. How does SUMO Mediate Sp3 Transcriptional Repression?

In attempting to find condition in which Sp3 SUMOylation may alter, we observed that the ratio between Sp3 unmodified and SUMOylated Sp3 is not significantly changed in different cell lines or mouse tissues (see Results-3.3 Is Sp3 regulated by SUMOylation?). Also, metabolic stress conditions, respective serum induction, serum starvation and heat shock were found not to alter Sp3 SUMOylation.

Nevertheless, there is no detectable alteration in SUMOylation pattern in mammalian cells treated with both, cycloheximide and TNF-alpha drugs and trichostatin A (TSA) treatment of insect cells does not affect the SUMOylation level of transiently transfected Sp3.

Treatment of cells with the proteasome inhibitor MG-132 dramatically reduces Sp3 SUMOylation level.

It was shown that PML, SUMO-1, Sp100, and proteasomes localized to the nucleoli in the proteasome inhibitor-treated cells with MG-132, suggesting that nucleoli may be involved in the regulation of proteasome-dependent protein degradation. The nucleolar accumulation may also reflect a natural turnover of these proteins that involves trafficking through the nucleoli (Mattsson et al., 2001). Contrary, another report shows that upon MG-132 treatment one can observe a slight increase in SUMOylation of PML and Sp100 (Everett et al., 1998).

However, the overall amount of Sp3 is not reduced upon advising that Sp3 is not degraded via proteasome-dependent pathway.

One possible explanation of altering of Sp3 SUMOylation could be that the MG-132 proteasome inhibitor prevents proteasome degradation of SUMO specific isopeptidase, which subsequently remove the Sp3-SUMO moiety. Ross and coworkers reported that the SUMO-1 protease SuPr-1 catalyzes the removal of SUMO-1 from Sp3 *in vivo* and stimulates transcriptional activation by Sp3 (Ross et al., 2002). On the other hand, I observed that upon MG-132 treatment of insect cells transfected with wt Sp3 and a corresponding reporter containing four Sp3 binding sites, the Sp3 transcriptional activation is not enhanced, suggesting that this proteasome inhibitor might be implicated in other steps of Sp3 SUMOylation.

While many explanations have been proposed for SUMO-mediated Sp3 transcriptional repression, a model must accommodate two apparently contradictory observations. Only a small proportion of Sp3 transcription factor appears to be

SUMOylated. However, under these conditions the Sp3 transcription factor is maximally repressed and mutation of the acceptor lysine for SUMO modification relieves repression. A convenient model for Sp3 transcription factor regulation by SUMO that accommodates these observations is outlined in Fig.4.2.

In this model newly synthesized Sp3 is rapidly SUMOylated and incorporated into a repression complex constituted in a SUMO-dependent fashion. Constitutively active SUMO-specific proteases can then catalyze removal of SUMO, but the transcription factor is retained in the repression complex (in a SUMO-independent fashion). Under normal circumstances a relatively slow dissociation of the stable repression complex would release sufficient unmodified Sp3 transcription factor to allow basal transcription. Thus, SUMO is required for the initiation of repression, but not for the maintenance of repression. The presence of enzymes involved in SUMO conjugation and deconjugation at the same sites within the cell nucleus (Zhang et al., 2002) suggest that SUMO modification is a highly dynamic process with substrates undergoing rapid SUMO modification and deconjugation. Also, expression of an Sp3 mutant, where the SUMO acceptor lysine has been altered, is transcriptionally active and refractive to SUMO-dependent repression.

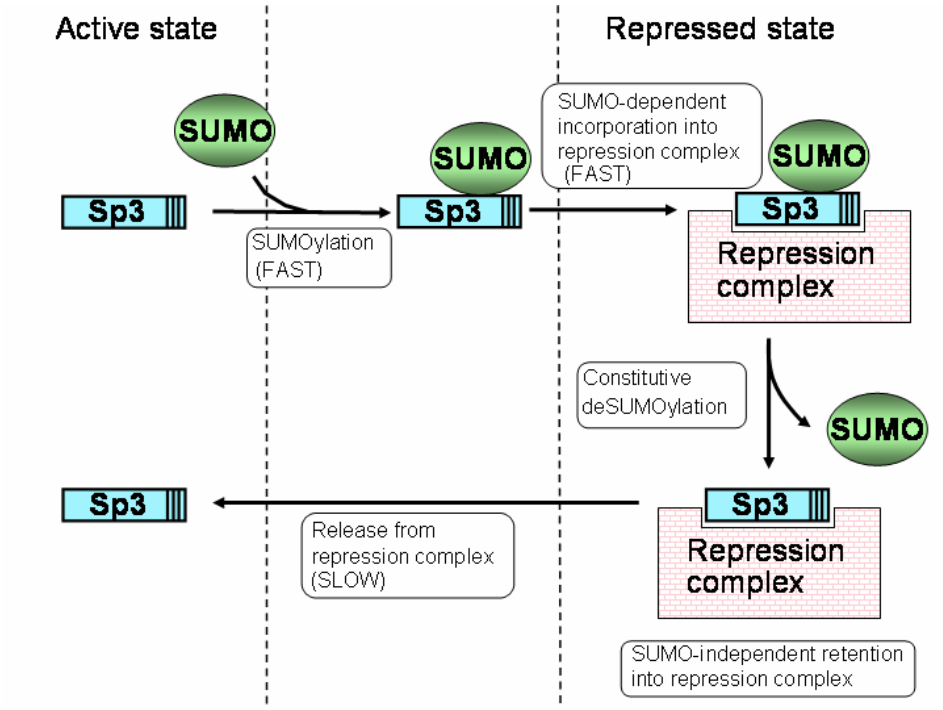


Fig. 4.2. Model for Sp3 transcriptional repression by SUMO. (Adapted after Girdwood et al. 2004)

Studies of the mechanisms by which SUMO modification regulates transcription-factor activity are complicated by the fact that in many cases not only the activator but also its associated coactivators, and/or corepressors, are modified by SUMO. It is possible that association of multiple SUMO-modified proteins in a complex may amplify effects of experimental alterations in the SUMOylation machinery or mask effects of mutation of individual SUMO acceptor lysines. If SUMO functions to promote interactions important for transcriptional repression, it may not matter exactly which subunit in a transcription factor complex is SUMO modified, so much as that SUMO is conjugated to one or more subunits in the complex. According to this view, it is modification of the complex *per se* that is important for regulation (Jackson, 2001). However, the mechanism(s) of transcriptional repression mediated by SUMOylation is still unclear.

4.7. Conclusion and Future Directions

The large and growing number of SUMO substrates that are now available for detailed studies demonstrates a previously unexpected diversity of physiological processes affected by this modification. Many of the SUMO-modified proteins identified to date are promoter-specific transcription factors, coactivators, or corepressors. Although the effects of SUMO modification on transcription factor activity are varied, in the majority of cases that have been described to date, attachment of SUMO appears to repress the activity of transcriptional activators (Gill, 2003; Verger et al., 2003).

The transcription factor Sp3, for example, has been shown to be SUMO modified *in vivo* and removal of SUMO by mutation of the Sp3 acceptor lysines or cotransfection with a SUMO protease dramatically increased transcriptional activity of Sp3 (Sapetschnig et al. 2002; Ross et al., 2002). These findings suggest that SUMO modification may contribute to the complex activity of Sp3, which has long been known to function as both an activator and a repressor of transcription dependent on the promoter context. Furthermore, Sp3 SUMOylation is one of the most recent found significant differences (Fig.4.3) between Sp1 and Sp3, considered for long time as redundant transcriptional regulators (Sapetschnig et al., 2004).

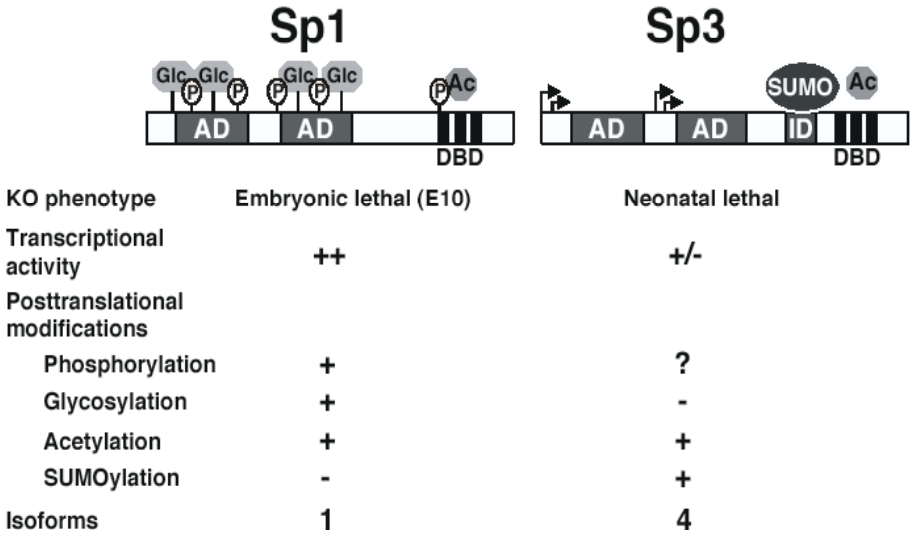


Fig. 4.3. Comparison of known structural, biological and biochemical features of the transcription factors Sp1 and Sp3. (Sapetschnig et al. 2004).

The only site of SUMO modification in Sp3 lies within a previously defined inhibitory domain. Similarly, the sites of SUMO attachment in some of the transcription factors, including C/EBP proteins, Elk-1, c-Myb, and steroid hormone receptors, have also been mapped to regions previously shown to function as inhibitory domains (Poukka et al. 2000; Abdel-Hafiz et al. 2002; Tian et al. 2002; Subramanian et al. 2003; Yang et al. 2003).

If the transcriptional responses (and many other regulatory events) require rapid activation and inactivation, it may be that the reversibility of SUMO addition and removal is critical for the dynamic assembly of regulatory complexes. Indeed, in some cases, SUMOylation might simply be a promiscuous process (intensified by substrate overexpression) that is normally kept in check by SUMO proteases, and for which a specific, SUMO-related function might not exist. On the other hand, it is also possible that, under physiological conditions (without artificial overexpression of the substrate and/or SUMO) the *bona fide* effects of SUMOylation might be more pronounced, or even quite different from what is observed using current experimental methods. Moreover, it must be considered that SUMOylation is a dynamic, reversible process, perhaps also explaining why, in many cases, only little or no modified protein can be detected under physiological conditions. Thus, SUMO modification may function to repress activation by many transcription factors.

How SUMOylation influence Sp3 transcriptional repression, and whether PIAS1 participate in the transcriptional repressor complex is also an interesting challenge for future studies. Analyzing transgenic animals expressing Sp3 isoforms and SUMOylation mutants could give important information concerning the function of individual Sp3 isoforms and furthermore the role of Sp3 SUMO modification in living animals. Identification and characterisation of Sp3- and Sp3-SUMO-associated proteins could unravel the prospect that Sp3-SUMO or non-modified Sp3 recruit enzymes that modify Sp3 itself or chromatin (potentially kinases, acetylases, deacetylases, methylases etc.). Moreover, addressing the function of a given Sp3/Sp3-SUMO-interacting protein by transient over-expression studies in various cell lines (SL2 cells, HeLa cells or fibroblast cell lines) is demanding. Vice versa, important informations about SUMO mediation of Sp3 transcriptional activity will also be obtained by knock-down the Sp3/Sp3-SUMO-interacting proteins using the siRNA technology.

5. References

Abdel-Hafiz, H., Takimoto, G. S., Tung, L., and Horwitz, K. B. (2002). The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. J Biol Chem *277*, 33950-33956.

Abraham, S. and. Solomon, W. B. (2000). A novel glutamine-rich putative transcriptional adaptor protein (TIG-1), preferentially expressed in placental and bone-marrow tissues. Gene *255 (2),* 389-400.

Adams, C.C. and Workman, J.L. (1993). Nucleosome displacement in transcription. Cell *72*, 305-308.

Angerer, N.D., Du, Y., Nalbant, D., Williams, S.C. (1999). A short conserved motif is required for repressor domain function in the myeloid-specific transcription factor CCAAT/enhancer-binding protein epsilon. J. Biol. Chem. *274*, 4147-4154.

Athanikar, J.N., Sanchez, H.B., Osborne, T.F. (1997). Promoter selective transcriptional synergy mediated by sterol regulatory element binding protein and Sp1: a critical role for the Btd domain of Sp1. Mol. Cell. Biol. *17*, 5195-5200.

Azuma, Y., Arnaoutov, A., and Dasso, M. (2003). SUMO-2/3 regulates topoisomerase II in mitosis. J Cell Biol *163*, 477-487.

Baeuerle, P.A. and Baltimore, D. (1988). Ikb: a specific inhibitor of the NF-kB transcription factor. Science *242*, 540-546.

Baldwin, A.S. Jr. (1996). The NF-kB and IkB proteins: new discoveries and insights. Annu. Rev. Immunol. *14*, 649-683.

Best, J. L., Ganiatsas, S., Agarwal, S., Changou, A., Salomoni, P., Shirihai, O., Meluh, P. B., Pandolfi, P.P., and Zon, L. I. (2002). SUMO-1 protease-1 regulates gene transcription through PML. Mol Cell *10*, 843-855.

Bies, J., Markus, J., and Wolff, L. (2002). Covalent attachment of the SUMO-1 protein

to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. J Biol Chem *277*, 8999-9009.

Birnbaum, M.J., van Wijnen, A.J., Odgren, P.R., Last, T.J., Suske, G., Stein, G.S., Stein, J.L. (1995). Sp1 transactivation of cell cycle regulated promoters is selectively repressed by Sp3. Biochemistry *34*, 16503-16508.

Blau, J., Xiao, H., McCracken, S., O'Hare, P., Greenblatt, J. and Bentley, D. (1996). Three functional classes of transcriptional activation domains. Mol. Cell. Biol. *16*, 2044-2055.

Boer, E. d., Rodriguez, P., Bonte, E., Krijgsveld, J., Katsantoni, E., Heck, A., Grosveld, F., and Strouboulis., J. (2003). Efficient biotinylation of tagged proteins by BirA biotin ligase in mammalian cells and transgenic mice. Proc Acad Sci *13*, 7480–7485.

Bonifacino, J. S and Weissman, A. M. (1998). Ubiquitinand the control of protein fate in the secretory and endocytic pathways. Annu. Rev. Cell Dev. Biol. *14*, 19–57.

Bouwman, P., Göllner, H., Elsässer, H. P., Eckhoff, G., Karis, A., Grosveld, F., Philipsen, S., and Suske, G.. (2000). Transcription factor Sp3 is essential for post-natal survival and late tooth development. EMBO J *19*, 655-661.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem *72*, 248-254.

Brasier A.R., Tate J.E., and Habener J.F. (1989). Optimized use of firefly luciferase assay as a reporter gene in mammalian cell lines. BioTechniques *7*,1116-1122.

Braun,H. and Suske,G.. (1999) Vectors for inducible expression of dual epitope-tagged proteins in insect cells. Biotechniques 26, 1038-1042.

Braun, H., Koop, R., Ertmer, A., Nacht, S., and Suske, G.. (2001). Transcription factor Sp3 is regulated by acetylation. Nucleic Acids Res *29*, 4994-5000.

Brown, H.J., Sutherland, J.A., Cook, A., Bannister, A.J., Kouzarides, T. (1995). An inhibitor domain in c-Fos regulates activation domains containing the HOB1 motif. EMBO J *14*, 124-131.

Calhoun, V.C., Stathopoulos, A. and Levine, M. (2002). Promoter-proximal tethering elements regulate enhancer-promoter specificity in the *Drosophila Antennapedia* complex. Proc. Natl. Acad. Sci. USA *99*, 9243-9247.

Ciechanover, A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J. *17*, 7151–7160.

Choudhury, B.K and Li, S. S. (1997). Identification and characterization of the SMT3 cDNA and gene from nematode *Caenorhabditis elegans*. Biochem. Biophys. Res. Commun. *234*, 788–791.

Courey, A.J. and Tjian, R. (1988). Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell *55*, 887-898.

Chen, A., Mannen, H., and Li, S.S. (1998). Characterization of mouse ubiquitin-like SMT3A and SMT3B cDNAs and gene/pseudogenes. Biochem. Mol. Biol. Int. *46*, 1161–1174.

Chung, C. D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., and Shuai, K. (1997). Specific inhibition of Stat3 signal transduction by PIAS3. Science *278*, 1803-1805.

Cull, M. G., and Schatz, P. J. (2000). Biotinylation of proteins in vivo and in vitro using small peptide tags. Methods Enzymol *326*, 430-440.

Dennig, J., Beato, M., and Suske, G.. (1996). An inhibitor domain in Sp3 regulates its glutamine-rich activation domains. Embo J *15*, 5659-5667.

Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998). SUMO-1 modification of Ikappa Balpha inhibits NF-kappaB activation. Mol Cell *2*, 233-239.

Ding, H., Benotmane, A.M., Suske, G., Collen, D., Belayew, A. (1999).Functional interactions between Sp1 or Sp3 and the helicase-like transcription factor mediate basal expression from the human plasminogen activator inhibitor-1 gene. J. Biol. Chem. 274, 19573-19580.

Everett, R. D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M., and Parkinson, J. (1998). The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol *72*, 6581-6591.

Freiman, R. N., and Tjian, R. (2003). Regulating the regulators. Lysine modifications make their mark. Cell *112*, 11-17.

Freshney, R.I. (1993) Culture of animal cells, a manual of basic technique, 3rd edition, Wiley-Liss.

Gill, G.. (2003). Post-translational modification by the small ubiquitin-related modifier SUMO has big effects on transcription factor activity. Curr Opin Genet Dev *13*, 108-113.

Girdwood, D. W., Tatham, M. H., and Hay, R. T. (2004). SUMO and transcriptional regulation. Semin Cell Dev Biol *15*, 201-210.

Gong, L., Millas, S., Maul, G., and Yeh, E. (2000). Differential regulation of sentrinized proteins by a novel sentrin-specific protease. J Biol Chem *275*, 3355-3359.

Gonzalez, G.A. and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell *59*, 675-680.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G.. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. EMBO J *18*, 6462-6471.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268*,

1766-1769.

Göllner, H., Dani, C., Phillips, B., Philipsen, S., and Suske, G.. (2001). Impaired ossification in mice lacking the transcription factor Sp3. Mech Dev *106*, 77-83.

Gross, M., Liu, B., Tan, J., French, F. S., Carey, M., and Shuai, K. (2001). Distinct effects of PIAS proteins on androgen-mediated gene activation in prostate cancer cells. Oncogene *20*, 3880-3887.

Hagen, G., Müller, S., Beato, M., and Suske, G.. (1992). Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes. Nucleic Acids Res *20*, 5519-5525.

Hagen, G., Müller, S., Beato, M., and Suske, G.. (1994). Sp1-mediated transcriptional activation is repressed by Sp3. EMBO J *13*, 3843-3851.

Halkidou, K., Logan, I.R., Cook, S., David E. N., and Robson C. N. (2004). Putative involvement of the histone acetyltransferase Tip60 in ribosomal gene transcription. J Cell Sci. 2004 *15*, 5847-5854.

Hampsey, M. and Reinberg, D. (2003). Tails of intrigue: phosphorylation of RNA polymerase II mediates histone methylation. Cell *113*, 429-432.

Hanania, U., Furman-Matarasso, N., Ron, M. and Avni, A. (1999). Isolation of a novel SUMO protein from tomato that suppresses EIX-induced cell death. Plant J. *19*, 533–541.

Hari, K. L., Cook, K. R., and Karpen, G. H. (2001). The Drosophila Su(var)2-10 locus regulates chromosome structure and function and encodes a member of the PIAS protein family. Genes Dev *15*, 1334-1348.

Hay,R.T. (2001) Protein modification by SUMO. Trends Biochem. Sci., *26*, 332-333.

Hershko, A and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Hicke, L. (1997). Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. FASEB J. *11*, 1215–1226.

Hietakangas, V., Ahlskog, J. K., Jakobsson, A. M., Hellesuo, M., Sahlberg, N. M., Holmberg, C. I., Mikhailov, A., Palvimo, J. J., Pirkkala, L., and Sistonen, L. (2003). Phosphorylation of Serine 303 Is a Prerequisite for the Stress-Inducible SUMO Modification of Heat Shock Factor 1. Mol Cell Biol *23*, 2953-2968.

Hilgarth, R. S., Murphy, L. A., Skaggs, H. S., Wilkerson, D. C., Xing, H., and Sarge, K. D. (2004). Regulation and function of SUMO modification. J Biol Chem *279*, 53899-53902.

Hochstrasser, M. (2000). Biochemistry. All in the ubiquitin family. Science *289*, 563-564.

Hochstrasser, M. (2000). Evolution and function of ubiquitin-like protein-conjugation systems. Nat. Cell Biol. *2*,E153–E157.

Hodges, M., Tissot, C., Freemont, P. S. (1998). Protein regulation: tag wrestling with relatives of ubiquitin. Curr. Biol. *8*, R749–752.

Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature *419*, 135-141.

Howe, K., Williamson, J., Boddy, N., Sheer, D., Freemont, P and Solomon, E. (1998). The ubiquitin homology gene PIC1: characterization of mouse (Pic1) and human (UBL1) genes and pseudogenes. Genomics *47*, 92–100.

Huang, T. T., Wuerzberger-Davis, S. M., Wu, Z. H., and Miyamoto, S. (2003). Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. Cell *115*, 565-576.

Huang, Y. P., Wu, G., Guo, Z., Osada, M., Fomenkov, T., Park, H. L., Trink, B.,

Sidransky, D., Fomenkov, A., and Ratovitski, E. A. (2004). Altered Sumoylation of p63a Contributes to the Split-Hand/Foot Malformation Phenotype. Cell Cycle *3*.

Jackson, S.P. and Tjian, R. (1988). O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. Cell *55*, 125-133.

Jackson, P. K. (2001). A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. Genes Dev *15*, 3053-3058.

Jentsch, S and Pyrowolakis, G.. (2000). Ubiquitin and its kin: How close are the family ties? Trends Cell Biol. *10*, 335–342.

Johnson, P., R and Hochstrasser, M. (1997). SUMO-1: Ubiquitin gains weight. Trends Cell Biol. *7*, 408–413.

Jordan M., Schallhorn A., and Wurm F.W. (1996). Transfecting mammalian cells: Optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res. *24*, 596-601.

Ihn, H., Trojanowska, M., (1997). Sp3 is a transcriptional activator of the human alpha2(I) collagen gene. Nucleic Acids Res. *25*, 3712-3717.

Iniguez-Lluhi, J.A., and Pearce, D. (2000). A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. Mol. Cell. Biol. *20*, 6040–6050.

Kadonaga, J.T. and Tjian, R. (1986). Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA *83*, 5889-5893.

Kadonaga, J.T., Carner, K.R., Masiarz, F.R. and Tjian, R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell *51*, 1079-1090.

Kadonaga, J. T. (2004). Regulation of RNA Polymerase II Transcription by Sequence-Specific DNA Binding Factors. Cell *116*, 247–257.

Kagey, M. H., Melhuish, T. A., and Wotton, D. (2003). The polycomb protein Pc2 is a SUMO E3. Cell *113*, 127-137.

Kahyo, T., Nishida, T., and Yasuda, H. (2001). Involvement of PIAS1 in the Sumoylation of Tumor Suppressor p53. Mol Cell *8*, 713-718.

Kamitani, T., Kito, K., Nguyen, H. P., Fukuda-Kamitani, T., and Yeh, E. T. (1998). Characterization of a second member of the sentrin family of ubiquitin-like proteins. J Biol Chem *273*, 11349-11353.

Kamitani, T., Yeh, E. T., Strauss, J. F., 3rd, and Maul, G..G.. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein Daxx to this nuclear structure when modified by SUMO-1. J Cell Biol *147*, 221-234.

Kennett, S.B., Udvadia, A.J., and Horowitz, J.M. (1997). Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. Nucleic Acids Res. *25*, 3110–3117.
Kim, K.I., Baek, S.H., and Chung, C.H. 2002. Versatile protein tag, SUMO: Its enzymology and biological function. J Cell Physiol *191*, 257–268.

Kim, J., Cantwell, C.A., Johnson, P.F., Pfarr, C. M., and Williams, S.C. (2002). Transcriptional activity of CCAAT/enhancer-binding proteins is controlled by a conserved inhibitory domain that is a target for sumoylation. J Biol Chem *277*, 38037-38044.

Kim, K.I., Baek, S.H., and Chung, C.H. (2002). Versatile protein tag, SUMO: its enzymology and biological function. J Cell Physiol *191*, 257-268.

Kingsley, C., and Winoto, A. (1992). Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. Mol Cell Biol *12*, 4251–4261.

Kotaja, N., Karvonen, U., Janne, O. A., and Palvimo, J.J. (2002a). The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1. J Biol

Chem *277*, 30283-30288.

Kotaja, N., Karvonen, U., Janne, O. A., and Palvimo, J.J. (2002b). PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol Cell Biol *22*, 5222-5234.

Lammer, D., Mathias, N., Laplaza, J.M., Jiang, W and Liu, Y. (1998). Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex. Genes Dev. 12, 914–926.

Laybourn, P.J. and Kadonaga, J.T. (1992). Threshold phenomena and long-distance activation of transcription by RNA polymerase II. Science *257*, 1682-1685.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science *240*, 1759-1764.

Le Drean, Y., Mincheneau, N., Le Goff, P., and Michel, D. (2002). Potentiation of glucocorticoid receptor transcriptional activity by sumoylation. Endocrinology *143*, 3482-3489.

Li, S.J. and Hochstrasser, M. (1999). A new protease required for cell-cycle progression in yeast. Nature *398***,** 246–251.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Gen *3*, 662-673.

Li, S.J. and Hochstrasser, M. (2003). The Ulp1 SUMO isopeptidase: Distinct domains required for viability, nuclear envelope localization, and substrate specificity. J. Cell Biol. *160,* 1069–1081.

Liu, B., Liao, J., Rao, X., Kushner, S. A., Chung, C. D., Chang, D. D., and Shuai, K. (1998). Inhibition of Stat1-mediated gene activation by PIAS1. Proc Natl Acad Sci USA *95*, 10626-10631.

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. Cell *88*, 97-107.

Majello, B., De Luca, P., Hagen, G., Suske, G.., and Lania, L. (1994). Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1. Nucleic Acids Res *22*, 4914-4921.

Majello, B., De Luca, P., Suske, G.., and Lania, L. (1995). Differential transcriptional regulation of c-myc promoter through the same DNA binding sites targeted by Sp1-like proteins. Oncogene *10*, 1841–1848.

Majello, B., De Luca, P., and Lania, L. (1997). Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. J. Biol. Chem. *272*, 4021–4026.

Mao, Y., Sun, M., Desai, S. D. and Liu, L. F. (2000). SUMO-1 conjugation to topoisomerase I: a possible repair response to topoisomerase-mediated DNA damage. Proc. Natl Acad. *Sci. 97*, 4046–4051.

Marin, M., Karis, A., Visser, P., Grosveld, F., Philipsen, S., (1997). Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell *89*, 619-628.

Mattsson, K., Pokrovskaja, K., Kiss, C., Klein, G., and Szekely, L. (2001). Proteins associated with the promyelocytic leukemia gene product (PML)-containing nuclear body move to the nucleolus upon inhibition of proteasome-dependent protein degradation. Proc Natl Acad Sci U S A *98*, 1012-1017.

Matunis, M. J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. J Cell Biol *135*, 1457-1470.

Melchior, F. (2000). SUMO–nonclassical ubiquitin. Annu. Rev. Cell Dev. Biol. *16*,

591–626.

Melchior, F., Schergaut, M., and Pichler, A. (2003). SUMO: ligases, isopeptidases and nuclear pores. Trends Biochem Sci *28*, 612-618.

Mermod, N., O'Neill, E.A., Kelly, T.J. and Tjian, R. (1989). The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. Cell *58*, 741-753.

McKenna, N.J. and O'Malley, B.W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. Cell *108*, 465-474.

Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000). Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. J Biol Chem *275*, 36316-36323.

Moon, H., Filippova, G., Loukinov, D., Pugacheva, E., Chen, Q., Smith, S. T., Munhall, A., Grewe, B., Bartkuhn, M., Arnold, R., Burke, L. J., Renkawitz-Pohl, R., Ohlsson, R., Zhou, J., Renkawitz, R., Lobanenkov, V. (2005) CTCF is conserved from Drosophila to humans and confers enhancer blocking of the Fab-8 insulator. EMBO Rep *6(2),* 165-70.

Moilanen A. M., Karvonen U., Poukka H., Yan W., Toppari J and Janne O. A. (1999) A testis-specific androgen receptor coregulator that belongs to a novel family of nuclear proteins. J. Biol. Chem. *274*, 3700–3704

Müller, S., Matunis, M.J., and Dejean, A. (1998). Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. EMBO J. *17*, 61–70.

Müller, S., Berger, M., Lehembre, F., Seeler, J. S., Haupt, Y., and Dejean, A. (2000). c-Jun and p53 activity is modulated by SUMO-1 modification. J Biol Chem *275*, 13321-13329.

Müller, S., Hoege, C., Pyrowolakis, G.., and Jentsch, S. (2001). SUMO, ubiquitin's mysterious cousin. Nat Rev Mol Cell Biol *2*, 202-210.

Muchardt, C., and Yaniv, M. (2001). When the SWI/SNF complex remodels...the cell cycle. Oncogene *20*, 3067-3075.

Murata, Y., Kim, H.G., Rogers, K.T., Udvadia, A.J., Horowitz, J.M. (1994). Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. J. Biol. Chem. 269, 20674-20681.

Muratani, M., Gerlich, D., Janicki, S. M., Gebhard, M., Eils, R., and Spector, D. L. (2002). Metabolic-energy-dependent movement of PML bodies within the mammalian cell nucleus. Nat Cell Biol *4*, 106-110.

Murre, C., McCaw, P.S. and Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell *56*, 777-783.

Narlikar, G.J., Fan, H.-Y. and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell *108*, 475-487.

Ohsumi, Y. (1999). Molecular mechanism of autophagy in yeast, *Saccharomyces cerevisiae*. Philos. Trans. R Soc. London Ser. B *354*, 1577–1580; discussion 80–81.

Oleksiak, M. F., and Crawford, D. L. (2002) 5' Genomic Structure of Human Sp3. Mol. Biol. Evol. *19*, 2026-2029.

Pabo, C.O. and Sauer, R.T. (1992). Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. *61*, 1053-1095.

Paranjape, S.M., Kamakaka, R.T. and Kadonaga, J.T. (1994). Role of chromatin structure in the regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. *63*, 265-297.

Philipsen, S., and Suske, G.. (1999). A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res *27*, 2991-3000.

Pichler, A., Gast, A., Seeler, J. S., Dejean, A., and Melchior, F. (2002). The nucleoporin RanBP2 has SUMO1 E3 ligase activity. Cell *108*, 109-120.

Pichler, A., and Melchior, F. (2002). Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport. Traffic *3*, 381-387.

Poukka, H., Karvonen, U., Janne, O. A., and Palvimo, J. J. (2000). Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci U S A *97*, 14145-14150.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods *24*, 218-229.

Ptashne, M. and Gann, A. (1997). Transcriptional activation by recruitment. Nature *386*, 569-577.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol *17*, 1030-1032.

Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. J Biol Chem *276*, 12654-12659.

Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999). SUMO-1 modification activates the transcriptional response of p53. EMBO J *18*, 6455-6461.

Ross, S., Best, J. L., Zon, L. I., and Gill, G. (2002). SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. Mol Cell *10*, 831-842.

Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001). PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. Genes Dev *15*, 3088-3103.

Saitoh, H., and Hinchey, J. (2000). Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J Biol Chem *275*, 6252-6258.

Sambrook, J., Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, 3nd edition. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sambrook, J., Fritsch, E. F. and Maniatis T (1989). Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Santoro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988). A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature *334*, 218-224.

Sapetschnig, A., Koch, F., Rischitor, G., Mennenga, T., and Suske, G.. (2004). Complexity of translationally controlled transcription factor Sp3 isoform expression. J Biol Chem *279*, 42095-42105.

Sapetschnig, A., Rischitor, G., Braun, H., Doll, A., Schergaut, M., Melchior, F., and Suske, G.. (2002). Transcription factor Sp3 is silenced through SUMO modification by PIAS1. Embo J *21*, 5206-5215.

Schmidt, D. and Müller, S. (2003). PIAS/SUMO: new partners in transcriptional regulation. CMLS, Cell. Mol. Life Sci. *60,* 2561–2574.

Seeler, J. S., Marchio, A., Sitterlin, D., Transy, C., and Dejean, A. (1998). Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. Proc Natl Acad Sci U S A *95*, 7316-7321.

Seeler, J. S., and Dejean, A. (1999). The PML nuclear bodies: actors or extras? Curr

Opin Genet Dev *9*, 362-367.

Seeler, J.S. and Dejean, A. (2001). SUMO: of branched proteins and nuclearbodies. *Oncogene 20*, 7243–7249.

Seeler, J. S., and Dejean, A. (2003). Nuclear and unclear functions of SUMO. Nat Rev Mol Cell Biol *4*, 690-699.

Shav-Tal, Y., Singer, R. H., and Darzacq X. (2004). Imaging gene expression in single living cells. Nature Reviews Mol Cell Biol *5*, 856-862.

Sjottem, E., Anderssen, S., Johansen, T. (1996). The promoter activity of long terminal repeats of the HERV-H family of human retrovirus-like elements is critically dependent on Sp1 family proteins interacting with a GC/GT box located immediately 3' to the TATA box. J. Virol. *70*, 188-198.

Sloper-Mould, K., Jemc, J., Pickart, C., and Hicke, L. 2001. Distinct functional surface regions on ubiquitin. J. Biol. Chem. *276*, 30483–30489.

Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., and Chen, Y. (2004). Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. Proc Natl Acad Sci U S A *101*, 14373-14378.

Spector, D., Goldman, R.R., and Leinwand, L.A. (1998) Cells: a laboratory manual. Cold Spring Harbor Laboratory Press.

Sternsdorf, T., Jensen, K., Reich, B., and Will, H. (1999). The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. J Biol Chem *274*, 12555-12566.

Su, K., Roos, M.D., Yang, X., Han, I., Paterson, A.J., Kudlow, J.E. (1999). An N-terminal region of Sp1 targets its proteasome dependent degradation in vitro. J. Biol. Chem. *274*, 15194-15202.

Subramanian, L., Benson, M. D., and Iniguez-Lluhi, J. A. (2003). A synergy control

motif within the attenuator domain of CCAAT/enhancer-binding protein alpha inhibits transcriptional synergy through its PIASy-enhanced modification by SUMO-1 or SUMO-3. J Biol Chem *278*, 9134-9141.

Suske, G., Bruford E. and Philipsen S. (2005). Mammalian SP/KLF transcription factors: bring in the family. Genomics, in press.

Suske, G.. (1999). The Sp-family of transcription factors. Gene *238*, 291-300.

Suzuki, T., Nakayama, T., Choo, D. W., Hirano, Y., Kurihara, T., Nishino, T., and Esaki, N. (2003). Cloning, heterologous expression, renaturation, and characterization of a cold-adapted esterase with unique primary structure from a psychrotroph Pseudomonas sp. strain B11-1. Protein Expr Purif *30*, 171-178.

Tan J. A., Hall S. H., Hamil K. G., Grossman G., Petrusz P. and French F. S. (2002). Protein inhibitors of activated STAT resemble scaffold attachment factors and function as interacting nuclear receptor coregulators. J. Biol. Chem. *277*, 16993– 17001.

Tanaka, K., Nishide, J., Okazaki, K., Kato, H and Niwa, O. (1999). Characterization of a fission yeast SUMO-1 homologue, Pmt3p, required for multiple nuclear events, including the control of telomere length and chromosome segregation. Mol. Cell. Biol. *19*, 8660–8672.

Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H., and Hay, R. T. (2001). Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. J Biol Chem *276*, 35368-35374.

Tian, S., Poukka, H., Palvimo, J. J., and Janne, O. A. (2002). Small ubiquitin-related modifier-1 (SUMO-1) modification of the glucocorticoid receptor. Biochem J *367*, 907-911.

Tussie-Luna, M. I., Bayarsaihan, D., Seto, E., Ruddle, F. H., and Roy, A. L. (2002). Physical and functional interactions of histone deacetylase 3 with TFII-I family proteins and PIASxbeta. Proc Natl Acad Sci U S A *99*, 12807-12812.

Udvadia, A.J., Templeton, D.J., Horowitz, J.M. (1995). Functional interactions between the retinoblastoma (Rb) protein and Sp family members: superactivation by Rb requires amino acids necessary for growth suppression. Proc. Natl. Acad. Sci. *92*, 3953-3957.

Vaheri, A. and Pagano, J.S. (1965) Infectious poliovirus RNA: A sensitive method of assay. Virology *27*, 434.

Van Hoy, M., Leuther, K.K., Kodadek, T. and Johnston, S.A. (1993). The acidic activation domains of the GCN4 and GAL4 proteins are not α helical but form β sheets. Cell *72*, 587-594.

Verger, A., Perdomo, J., and Crossley, M. (2003a). Modification by SUMO. A role in transcriptional regulation. EMBO Rep *4*, 137-142.

Watts, F. Z. (2004). SUMO modification of proteins other than transcription factors. Semin Cell Dev Biol *15*, 211-220.

Wimmer, E.A., Frommer, G., Purnell, B.A., Jackle, H., (1996). Buttonhead and D-Sp1: a novel Drosophila gene pair. Mech. Dev. *59*, 53-62.

Yang, X., Zhang, F. and Kudlow, J.E. (2002). Recruitment of *O*-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein *O*-GlcNAcylation to transcriptional repression. Cell *110*, 69-80.

Yang, A., Kaghad, M., Caput, D. and McKeon, F. (2002). On the shoulders of giants: p63, p73 and the rise of p53. Trends Genet. *18*, 90-95.

Yang, S. H., Jaffray, E., Senthinathan, B., Hay, R. T., and Sharrocks, A. D. (2003). SUMO and transcriptional repression: dynamic interactions between the MAP kinase and SUMO pathways. Cell Cycle *2*, 528-530.

Yieh, L., Sanchez, H.B., Osborne, T.F. (1995). Domains of transcription factor Sp1 required for synergistic activation with sterol regulatory element binding protein 1 of low density lipoprotein receptor promoter. Proc. Natl. Acad. Sci. *92*, 6102-6106.

Yin, D. X., Zhu, L., and Schimke, R. T. (1996). Tetracycline-controlled gene expression system achieves high-level and quantitative control of gene expression. Anal Biochem *235*, 195-201.

Young, R.A. (1991). RNA polymerase II. Annu. Rev. Biochem. 60, 689-715.

Zhang, H., Saitoh, H., and Matunis, M. J. (2002). Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex. Mol Cell Biol *22*, 6498-6508.

Zhang, W., Shields, J. M., Sogawa, K., Fujii-Kuriyama, Y., and Yang, V. W. (1998). The gut-enriched Kruppel-like factor suppresses the activity of the CYP1A1 promoter in an Sp1-dependent fashion. J Biol Chem *273*, 17917-17925.

Zhang, X. L., Simmen, F. A., Michel, F. J., and Simmen, R. C. (2001). Increased expression of the Zn-finger transcription factor BTEB1 in human endometrial cells is correlated with distinct cell phenotype, gene expression patterns, and proliferative responsiveness to serum and TGF-beta1. Mol Cell Endocrinol *181*, 81-96.

Zhong, S., Muller, S., Ronchetti, S., Freemont, P. S., Dejean, A., and Pandolfi, P. P. (2000). Role of SUMO-1-modified PML in nuclear body formation. Blood *95*, 2748-2752.

Zhong, S., Salomoni, P., and Pandolfi, P.P. (2000). The transcriptional role of PML and the nuclear body. Nat. Cell Biol. *2*, E85–90.

Acknowledgements

Firstly I would like to express my profound gratitude to Prof. Dr. Guntram Suske for offering me the opportunity to work in his team as a PhD student on a captivating and intriguing topic, and for his full support through suggestions and active debates concerning the scientific research. Also, cordial thanks for offering me outstanding working conditions at the Institute of Molecular Biology and Tumor Research in Marburg, Germany.

I would like to thank Prof. Dr. Renate Renkawitz-Pohl and Prof. Dr. Michael Bölker for the permanent encouragements and stimulative scientific discussions.
Here I wish to express my gratefulness to Prof. Dr. Reinhard Fischer for supporting me constantly ever since we met and for his confidence in all my actions.

I would like to say a sincere "Danke schön", for the nice and friendly atmosphere given to the lab to all my colleagues, Alexandra Sapetschnig, Imme Krueger, Marion Vollmer, Bastian Stielow and Frank Baur and for helping with correction of my thesis.

To formerly lab colleagues, Dr. Heike Göllner and Dr. Harald Braun, my deep recognition for being always beside me with small tips and tricks, which made the life in the lab agreeable.

I am also deeply appreciative to Iris Rohner for the kindhearted and constant technical support and I would like to thank Karin Theis and Bernhard Gross for their friendship and help and to Ralf Klockgeter for assistance on computing problems.

My special thanks to Prof. Dr. Hans-Peter Elsässer and Dr. Daniel Ursu for real support with microscopy. Thanks too should be directed to Dr. Wolfgang Meissner for his kindness showed during size exclusion chromatography experiments.

My truthful appreciation to some of my formerly faculty colleagues, Simona Ursu, Anca Azoitei, Dr. Costel Darie, Dr. Ninel Azoitei, Dr. Gottfried Eisner, Dr. Cristian Cojocariu and Eugen Urzica for all those interesting discussions about my projects and not only, and for the friendly time spent together in Germany.

My bottomless gratitude belongs to my family, to my parents who always encouraged me in taking new prospects in life and offered me their tireless care, and to my wife Patricia, who is sharing with me the life around and outside the "biological" world.

Curriculum Vitae

Name	Grigore Rischitor
	Rudolf-Bultmann Str. 4
Address	35039 Marburg
	e-mail: rischitor@IMT.Uni-Marburg.de

Birth date, birth place	13. 10. 1969, Negresti, Romania
Family status	Married, no children

Education

08/2001-Present	PhD student, Prof. Dr. Guntram Suske Group, Institute for Molecular Biology and Tumor Research Marburg, Germany
10/1995-07/199	MS, Biology, Al.I. Cuza University, Iasi, Romania
10/1990-07/1995	BS, Biology, Al.I. Cuza University, Iasi, Romania
06/1988	General Qualification for University Entrance ("Baccalaureate"), Sanitary High School Suceava, Romania

Positions held

05/1997-11/2000	Biologist, specialist in investigation, conservation and restoration of patrimony objects, Regional Laboratory of Conservation-Restoration within "MOLDOVA" National Complex of Museums, Iasi, Romania
08/1995-05/1997	Biologist, Research Assistant in Applied Genetics, Research Department of Fishery Research Station „ACVARES", Iasi, Romania
01/1988-09/1990	Medical social worker (male nurse) in the field of sanitary hygiene, County Hygiene Centre –Vaslui, Romania

Marburg, Germany, February 2005