Complexity of Translationally Controlled
Transcription Factor Sp3 Isoform Expression

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Running Title: Sp3 isoforms
Sp3 is a ubiquitous transcription factor closely related to Sp1. Both proteins contain a highly conserved DNA-binding domain close to the C-terminus and two glutamine-rich domains in the N-terminal moiety. Immunoblot analyses of Sp3 reveal a striking complex protein pattern of up to eight distinct species. This pattern is not observed in Sp3-deficient cell lines showing that all signals reflect Sp3 antigen. In this study, we have unraveled the complexity of Sp3 expression. We show that four isoforms of Sp3 that retain different parts of the amino terminus are expressed in vivo. The four isoforms derive from alternative translational start sites at positions 1, 37, 856 and 907. An upstream open reading frame located at position -47 to -18 regulates expression of the two long isoforms. Unlike Sp1, none of the Sp3 isoforms is glycosylated. However, all four isoforms become SUMO-modified in vivo and in vitro specifically and exclusively at lysine residue 551. The transcriptional activity of the two long isoforms strongly depends on the promoter settings whereas the small isoforms appear to be inactive. The transcriptional activity of all the Sp3 isoforms is regulated by SUMO modification. Our results demonstrate that Sp3 has many unique features and is not simply a functional equivalent of Sp1.
INTRODUCTION

The transcription factor Sp3 is a ubiquitously expressed member of the Sp family of transcription factor that is involved in the expression and regulation of many genes including house keeping genes, tissue-specifically expressed genes, viral genes as well as cell cycle-regulated genes (1,2). Sp3 contains a highly conserved DNA-binding domain close to the C-terminus, and two glutamine-rich activation domains in the N-terminal moiety. The expression pattern, the structure as well as the DNA-binding properties of Sp3 are very similar to Sp1, which suggested originally that these two proteins exert similar functions. The physiological roles of Sp1 and Sp3, however, appear to be significantly different. Sp1 knockout mouse embryos are severely retarded in growth, and die after day 10 of embryonic development (E10) (3). Sp3-deficient embryos develop until birth, but die invariably of respiratory failure immediately after birth (4). In addition, late tooth and bone developmental processes are impaired in Sp3-/- mice (4,5).

Functional analyses of the transcriptional properties of Sp1 and Sp3 also revealed significant differences between these two transcription factors (6). On many reporter constructs containing multiple Sp-binding sites Sp3 is, unlike Sp1, inactive or acts only as a weak activator (7). The molecular basis for the inactivity of Sp3 under these conditions has been mapped to an inhibitory domain located between the second glutamine-rich activation domain and the zinc finger region (8). More recently, it was shown that Sp3 is posttranslationally modified by the Small Ubiquitin-like MOdifier
(SUMO) within its inhibitory domain, and that SUMO modification leads to inactivation (9,10).

All previously published studies with Sp3 (more than 500 citations in Medline) were performed with N-terminally truncated versions of Sp3. In addition, unraveling the transcriptional role of Sp3 was complicated by the fact that several isoforms exist (2,11).

For long it was thought that full-length Sp3 protein is derived from translational initiation at a non-AUG codon, whereas two small Sp3 isoforms derive from internally initiated translation at AUG start sites (11). However, recently, human genomic DNA sequences were identified that encompass three exons coding for additional 85 N-terminal amino acids of Sp3 (12).

Here, we report the cloning of the full-length Sp3 open reading frame that starts with a classical AUG initiation codon. The full-length Sp3 cDNA enabled us to unravel the complexity of the Sp3 antigen pattern observed in immunoblots. We show that in vivo four isoforms of Sp3 are expressed 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. None of the Sp3 isoforms is glycosylated. However, all four isoforms become SUMO-modified in vivo as well as in vitro specifically and exclusively at lysine residue 551. The two long isoforms of Sp3 can act as transcriptional activators on certain promoter settings whereas the two small isoforms appear to be always inactive. Mutational analyses of the SUMO acceptor site in the context of
different isoforms show that their transcriptional inactivity is regulated by SUMO modification. Our results demonstrate that Sp3 has many unique molecular features and is not simply a functional equivalent of Sp1.

**EXPERIMENTAL PROCEDURES**

*Plasmids*- The missing 5´-part of the Sp3 cDNA was cloned by RT-PCR of HeLa RNA using the primers 5´-actcggaattcCCTTTTGTGTTTCCCCGCACAGTCA-3´ and 5´-CTGTGCAGAAGCCAAATCACCTGT-5´. The resulting 470 bp product was cut with EcoRI (artificial site at the 5´-end of the forward primer) and NotI (site within the Sp3 cDNA sequence) and cloned into EcoRI/NotI-restricted pSPT18-Sp3 plasmid that contained the original published Sp3 sequences (13,14) For *in vitro* transcription/translation assays of wild-type Sp3 and mutants, the T7/Sp6 promoter containing pSPT18 plasmid was used. Transient expression of Sp3 variants in *Drosophila* Schneider SL2 cells (15) was driven by the *Drosophila* actin 5C 5´-flanking region. For transient expression of Sp3 variants in mammalian cells the CMV promoter containing pN3, pMCS-HA and pHA-MCS plasmids were used. pN3 was constructed by removal of the GFP moiety from pEGFP-N3 (Clontech) with BamHI and NotI. The plasmids pMCS-HA and pHA-MCS (16) are derivatives of pEGFP-N1 and pEGFP-C1 (Clontech), respectively, in which the GFP part of pEGFP is replaced by the HA epitope. Point mutations were introduced into pSPT18-Sp3 using the QuikChange XL site
directed mutagenesis kit (Stratagene). Mutagenised Sp3 cDNAs were subsequently recloned into pPac, pN3, pMCS-HA or pHA-MCS vectors. Detailed information on the construction of individual plasmids will be provided upon request.

**Cell culture and transient transfection assays**—For activation studies SL2 cells were transfected by the calcium phosphate method as described (15). Every plate received 4 µg of reporter plasmid (BCAT-2 or pGL3) and 2 µg of the β-galactosidase expression plasmid p97b as internal reference. Variable amounts of pPacSp3 expression plasmids were compensated with the plasmid pPac. 24 h after addition of DNA, the medium was changed and 24 h later the cells were washed twice with PBS prior to harvest. Chloramphenicol acetyltransferase (CAT) enzyme expression was assayed by ELISA according to the manufacturers instructions (Roche). Luciferase and β-galactosidase assays were carried out as described (17,18). Expression of CAT or luciferase enzymatic activity was normalized to β-galactosidase activity. Each transfection was repeated at least three times. For analyses of Sp3 isoforms SL2 and mammalian cells were transfected with the FuGEN6 reagent according to the manufacturers instructions (Roche). Lysate preparation was as described (10).

**Immunofluorescence and microscopy**—Stable transfected Sp3-/- KO MEFs or SL2 cells were plated on 13mm Ø round coverslips in 24 well tissue culture plates and 24 h post-transfection (SL2 cells) fixed for 25 min at room temperature with 4% paraformaldehyde in PBS. Cells were washed
twice for 5 min with PBS and subsequently permeabilized with 0.2% Triton-X100 in PBS for 15 min. After blocking with 3% bovine serum albumin in PBS for 1 h, cells were incubated with rabbit anti-Sp3 (Santa Cruz) for 1 hr at room temperature. Coverslips were washed three times for 5 min with PBS and incubated with a FITC- (Jackson Immunoresearch) or Alexa Fluor® 594- (Molecular Probes) conjugated secondary goat anti-rabbit IgG antibody for 1 h at room temperature in a dark cabinet. After three washings with PBS for 5 min, coverslips were mounted onto glass slides using Vectrashield mounting media with 4,6-diamino-2-phenylindole (DAPI) and sealed with nail polish. Fluorescence images were obtained on Leica DMLB microscope with Leica N PLAN 100X/1.25 oil objective.

**Immunoblotting**—Proteins resolved by SDS-PAGE were electro-transferred to PVDF membranes and probed with the appropriate antibodies. The following antibodies with the indicated dilutions in 20 mM Tris/HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20, 1% skim milk were used: Rabbit anti-Sp3 polyclonal IgG (Santa Cruz) 1:2000; rabbit anti-HA (Santa Cruz) 1:2000; rabbit anti-Sp1 polyclonal IgG (7) 1:5000, rat anti-HA monoclonal IgG (Roche) 1: 2000, horse radish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and HRP-conjugated goat anti-rat IgG (Amersham Biosciences) 1:10000. The enhanced chemoluminescence detection system (Amersham Biosciences) was used to visualize the peroxidase reaction.
**In vitro transcription/translation and autoradiography**- For in vitro transcription/translation of Sp3 proteins the TNT T7-coupled reticulocyte lysate system (Promega) was used according to the manufacturers instructions. Radioactive labeled reaction products were separated through 5% SDS polyacrylamide gels. Gels were subsequently treated with fixing solution (10% acetic acid, 25% 2-propanol) and Amplify reagent (Amersham Biosciences) prior to drying and exposure to X-ray films (Kodak).

**In vitro SUMOylation assay**- SUMO modification reactions of in vitro transcribed/translated Sp3 proteins were carried out at 30°C for 90 min in a total volume of 20 µl reaction buffer (20 mM Hepes, pH 7.3, 110 mM K-acetate, 2mM Mg-acetate, 0.5 mM EGTA, 0.05% Tween20, 0.4 mg/ml ovalbumin, 1 mM ATP, 5 mM DTT) containing 5 µl 35S-labeled substrate protein, 25 ng E1 enzyme (Aos1/Uba2), 75 ng E2 enzyme (Ubc9), 50 ng E3 ligase (GST-PIAS1) and 200 ng SUMO-1 (10). Reactions were stopped by adding 2x SDS Laemmli buffer (Sigma).

**Wheat germ agglutinin affinity chromatography (WGA)**- 293 cells expressing endogenous Sp proteins or transfected SL2 cells expressing Sp1, Sp3WT or the Sp3K551R mutant were harvested in lysis buffer (40 mM Tris/HCl, pH 6.8, 1% SDS, 2.5% β-mercaptoethanol, 0.2 mM PMSF and protease inhibitor cocktail). 100 µl of whole cell extracts (approximately 1 mg protein) was diluted 1:10 in binding buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.2 mM PMSF, protease inhibitor
cocktail) and incubated with 50 µl equilibrated agarose wheat germ lectin (Amersham Biosciences) for 1 hour at 4°C. Matrix-bound glycosylated proteins were washed extensively in binding buffer before adding 2x SDS Laemmli buffer (Sigma). For competition experiments, 100 mM N-acetyl D-glucosamine (Roth) was added to the binding buffer. Binding of Sp proteins to agarose wheat germ lectin was analyzed by Western blotting.
RESULTS

Complexity of Sp3 isoforms—High resolution immunoblot analyses with antibodies directed to the C-terminus of Sp3 resulted in a striking complex protein pattern. Four distinct proteins, two slow migrating of more than 100 kDa and two fast migrating species of approximately 72 kDa (Fig. 1A, lane 5) are observed in nuclear extracts or whole cell extracts prepared with RIPA buffer. Additional bands appear, when cells were lysed directly in SDS containing buffer. Under these conditions 7 to 8 Sp3 species are visualized in immunoblots (Fig. 1A, lanes 3, 4 and 6). All signals are specific for Sp3 since extracts prepared from Sp3-deficient embryonic stem cells did not cause any background (Fig. 1A, lane 2).

In the past, analyses that could clarify the nature of the various Sp3 species were hampered due to the lack of a full-length Sp3 cDNA clone. The original Sp3 cDNA sequences (13,14) did not contain an AUG translational start codon and it was postulated that translation of the Sp3 mRNA starts at an non-AUG codon (14). In the course of a comparative sequence analysis among the evolutionary related Sp transcription factors Sp1, Sp2, Sp3 and Sp4 (19) it was suggested that the 5′-end of Sp3 is absent in the published cDNA clones. Moreover, human genomic DNA sequences were identified that encompass three exons coding for additional 85 N-terminal amino acids of Sp3 (12). Based on these data, we cloned the missing 5′-part of Sp3 by RT PCR with primer pairs specific for sequences in exon 1 and exon 3 (GenBank accession number AF494280) (12) and
fused it to the Sp3 coding region. The resulting Sp3 cDNA codes for 781 amino acids when translated from the first AUG codon.

Expression of the four Sp3 isoforms observed in immunoblots can be reconstituted in a coupled in vitro transcription/translation assay using the full-length Sp3 cDNA clone (Fig. 1B, lane 2). Moreover, the additional Sp3 species observed exclusively in SDS-lysed cell extracts can also be reconstituted when in vitro translated Sp3 proteins were subjected to an in vitro SUMO modification reaction (10) (Fig. 1B, lane 1). This result strongly suggests that the additional signals observed in SDS-lysed cell extracts represent SUMO-modified versions of the four Sp3 isoforms observed in non-denatured nuclear extracts. Very likely, highly active isopeptidases present in nuclear extracts readily cleave the isopeptide bond between SUMO and Sp3 unless special precautions are taken to inactivate these enzymes.

All four Sp3 isoforms are derived from alternative translational start sites-The entire Sp3 coding region contains four AUG in frame codons at position 1, 37, 856 and 907 relative to the first AUG. To analyze whether the four isoforms (designated Sp3li-1, Sp3li-2, Sp3si-3 and Sp3si-4) observed in vitro derive from these potential translational initiation sites we mutated each of the four AUGs in the context of the full-length Sp3 cDNA (Fig. 2A). Each AUG mutation resulted in the lack of the appropriate isoform in an in vitro transcription/translation system (Fig. 2B, lanes 3, 4, 5 and 6). Thus the four different isoforms derive from translational initiation at the four AUG sites leading to translation products of 781, 769, 496 and
479 amino acids that differ in their N-terminal end (Fig 2C). We also analyzed an Sp3 cDNA mutant in which the first two AUGs were mutated (Fig 2B, 1.+2.AUGm in lane 8). In addition to the two small isoforms Sp3si-3 and Sp3si-4, this mutant produced several new long protein products not synthesized from the wild-type cDNA. We speculate that these proteins arise from non-AUG initiation codons. Inspection of the cDNA sequence revealed indeed the presence of three potential non-AUG initiation codons in a strong context (gacGUGga at position 52, gcgGUGg at position 133 and gauUUGg at position 289). We also asked whether expression of any of the smaller isoforms is dependent on the context of the first AUG codon. The wild-type sequence acuAUGa was replaced by an optimal Kozak sequence (gccAUGg). In vitro, this mutation did not impair expression of any of the four isoforms (Fig. 2B, lane 7), which is in contrast to the in vivo situation presented below.

Next we asked whether expression of the four Sp3 isoforms could be reconstituted in vivo after transfection of appropriate expression constructs. We cloned the full-length Sp3 cDNA and mutants into CMV promoter-driven expression vectors (pN3 and pMCS-HA vectors, see Experimental Procedures for details) useful for mammalian cells as well as in actin promoter-driven expression vectors (pPac) useful for insect Schneider cells (SL2 cells). After transfection of the wild-type Sp3 cDNA both long isoforms of Sp3 were expressed in 293 cells and SL2 cells similar to the situation in vitro. The two small isoforms of Sp3 were produced in 293 cells at low level but were not detectable in extracts from transfected SL2 cells (Fig. 2D and E). It should be noted that SUMO-modified isoforms
were observed in all cases where SDS extracts were prepared for Sp3 expression analyses (Fig. 2D). In those cases where we generated nuclear extracts, these posttranslational modified proteins were not observed (Fig. 2E) since hydrolases presumably cleaved the Sp3-SUMO isopeptide bond.

Mutation of any of the four AUG codons in the context of the full-length cDNA resulted in the absence of the appropriate isoform in 293 cells (Fig. 2 D, lanes 2, 4, 7 and 8). In SL2 cells mutation of the first and second AUG codon also resulted in the lack of the corresponding isoform (Fig 2E left, lanes 3 and 5). These results strongly suggest that in vivo the four isoforms of Sp3 derive also from alternative usage of the four in frame AUG start codons present in the Sp3 mRNA.

Additional point and 5΄-deletion mutants support this conclusion. Deletion of the 5΄-non-coding sequences and the first AUG codon (Δ1.AUG) resulted in the absence of the Sp3li-1 isoform whereas expression of the second Sp3 isoform was unchanged (Fig 2E, lane 7). Deletion of the first 85 codons (Δ1.+2.AUG in Fig 2E, lane 6) resulted in exclusive expression of the two small isoform (predominantly of Sp3si-4). Upon mutation of the first two AUG codons in the context of full-length Sp3 (1.+2.AUGm in Fig. 2D, lane 5) as well as upon deletion of 5΄-non-coding sequences and the first AUG and simultaneous replacement of the second AUG by AGC (Δ1.AUG/2.AUGm, in Fig 2E, lane 8) several new long protein products were observed. Similar to the in vitro situation, these new proteins likely arise from non-AUG start codons.

The constructs used in the above analyses did not contain the full 3΄-non-coding sequences. To test the influence of the 3΄-non-coding
sequences on the expression of different isoforms, we generated constructs with the complete 3′-untranslated region. The ratio of the four isoforms was not altered by the presence of the 3′-non-coding sequences neither in vitro nor in vivo (data not shown).

Expression of the long isoforms is regulated by an upstream open reading frame-During the course of our studies, we realized that the 5′-non-coding region of the Sp3 mRNA contains a short upstream open reading frame (uORF) at position –47 to –18 (Fig. 3A). Since uORFs can participate in translational control (20), we asked whether the Sp3mRNA uORF is involved in the regulation of Sp3 isoform expression. At first, we generated two Sp3 cDNA mutants. In one of them the uORF initiation site was replaced by UAG (uAUGm) while in the other the uAUG, normally in a suboptimal context, is placed in an optimal Kozak sequence (uAUGoK) (Fig. 3B). These mutations did not alter Sp3 isoform expression in an in vitro transcription/translation assays (data not shown). In contrast, upon transfection in SL2 or 293 cells, the uAUGm mutation almost entirely abolished translation from the second AUG and enhanced initiation from the first AUG (Fig. 3C, lane 2 and Fig. 3D, lane 3). Vice versa, upon increasing the uAUG strength, the Sp3 isoform 1 and 2 ratio shifted towards the second isoform (Fig. 3D, lane 4, uAUGoK). Expression of the small isoforms of Sp3 were not significantly altered by these mutations.

We also asked whether initiation site selection is dependent on the sequence of the uORF. The wild-type codons of the uORF were replaced by codons for glycine residues without changing the length of the uORF
(PolyG mutant in Fig. 3B). Compared to wild-type, this mutation did not alter the ratio of the two long isoforms (Fig. 3D, lane 8). In conclusion, the peptide that might be translated form the uORF is not involved in initiation site selection. We also generated mutants in which the distance of the uORF stop codon to the first AUG start codon was increased by 17 or 35 nucleotides (insertion mutants In-1 and In-2 in Fig. 3B). These mutations shifted expression of the long Sp3 isoforms towards the first isoform (Fig. 3D, lanes 6 and 7). Hence, the distance of the uORF to the first AUG start codon appears to be important for initiation site selection.

The SUMOylation motif present exclusively in the long isoform of Sp3 is not a target for SUMO modification—Sp3 is posttranslationally modified by SUMO at lysine 551 within the inhibitory domain of Sp3 (10). Close inspection of the N-terminal amino acid sequence revealed another potential site for SUMOylation present exclusively in the first Sp3 isoform (Fig. 4A). Lysine residue K9 lies within the sequence VKQE (amino acids 8-11) that fits well with the general SUMO conjugation motif ΨKXE (10,21). Obviously, an intriguing idea would be that the first and the second long Sp3 isoforms differ in their capacity to become posttranslationally modified by a SUMO moiety at the N-terminus. We asked whether lysine 9 is a target for SUMO modification in vivo or in vitro. Full-length versions of wild-type Sp3 or the K551R mutant either HA-epitope tagged at the N-terminus or the C-terminus were transfected into Sp3/- MEFs and 293 cells, and subjected to immunoblot analyses. SUMO-modified Sp3, visualized as a slower migrating species, was detected with the wild-type
Sp3 construct but not with the Sp3K551R construct (Fig. 4B). This result strongly suggests that exclusively K551 becomes SUMO-modified but not K9 or any other lysine residue of the Sp3 sequence. This conclusion is supported by results obtained in vitro. In vitro translation products of wild-type Sp3 as well as of the Sp3Δ1.AUG mutant lacking the first isoform of Sp3 but not of the Sp3K551R mutant are targets for SUMOylation in vitro (Fig. 4C). All together, these results show that lysine 9 within the VKQE motif that is only present in the longest Sp3 isoform is not a target for SUMO modification, neither in vivo nor in vitro.

Unlike Sp1, Sp3 is not glycosylated-The transcription factor Sp1 is posttranslationally modified by glycosylation (22). Since Sp3 is evolutionary and structurally very closely related to Sp1, we asked whether any of the isoforms of Sp3 is also modified by glycosylation. Glycosylated proteins bind strongly to wheat germ agglutinin (WGA) and WGA affinity chromatography has been used in the past as a major step to purify Sp1 from cell extracts (23).

Whole cell extracts of untransfected 293 cells and of SL2 cells transfected with Sp1, wild-type Sp3 or the SUMOylation-deficient Sp3K551R mutant were incubated with wheat germ agglutinin matrix. Subsequently, bound proteins were subjected to immunoblot analyses using antibodies to Sp1 and Sp3. As expected, endogenous Sp1 present in 293 extracts (Fig. 5, lane 2) as well as transiently (Fig. 5, lane 16) or stably (not shown) expressed Sp1 in SL2 cells bound strongly to wheat germ agglutinin (Fig. 5). Binding was specific as it was competed by an excess
of N-acetyl D-glucosamine. (Fig. 5, lane 4). Surprisingly, neither the small, the long nor the SUMO-modified isoforms of Sp3 bound to wheat germ agglutinin (Fig. 5, lanes 6 and 14). The same holds true for the SUMO modification-deficient Sp3K551R mutant (Fig. 5, lane 12). These results clearly show that the transcription factor Sp3, unlike Sp1, is not a target for posttranslational modification by glycosylation.

Functional differences between the various isoforms—All published data on Sp3 activation capacity were performed with N-terminally truncated versions of Sp3. Thus it was obvious to ask whether full-length Sp3 or the various isoforms of Sp3, respectively, differ in their capacity to activate transcription. The transcriptional activity of various Sp3 isoforms and mutants was analyzed by transiently transfecting Sp3 expression constructs along with reporter constructs in Schneider insect cells that lack endogenous Sp transcription factors. Gel shift experiments showed that all Sp3 isoforms bind GC-boxes specifically (Fig. 6A and data not shown). The BCAT-2 reporter contains two GC-boxes fused to the E1b TATA box. Upon transfection of the wild-type Sp3 construct or cDNA mutants from which predominantly the first isoform (2.AUGm), the second isoform (1.AUGm) or exclusively the third and fourth isoforms (Δ1.+2.AUG) were expressed, the BCAT-2 reporter became not activated (Fig. 6B). This result shows that all four isoforms of Sp3 are unable to activate the BCAT-2 reporter.

The SV40 promoter bearing five GC boxes becomes activated upon co-transfection of the wild-type Sp3 construct (WT) as well as by mutants
from which predominantly the first isoform (2.AUGm) or the second isoform (1.AUGm) are expressed. The small isoforms of Sp3 expressed upon transfection of the Δ1.+2.AUG construct did not activate this reporter construct (Fig. 6B).

The promoter-specific activation capacity of the long isoforms of Sp3 (781 and 769 aa) basically reiterate results obtained previously with an N-terminally tagged truncated version (697 aa) (8). Thus the two long isoforms of Sp3 do have the capacity to activate transcription from certain promoters, for instance the SV40 promoter, but are inactive on other promoter settings exemplified here by BCAT-2. In contrast, both promoters were not activated by the small Sp3 isoforms.

The capacity of Sp3 to activate transcription is silenced by SUMO modification at lysine K551 (9,10). These studies also have been performed with N-terminally truncated, epitope-tagged versions of Sp3 that, according to the results described above, mimicked the activity of the long isoforms. Since all four isoforms of Sp3 are SUMO-modified, we asked whether the inactivity of the full-length Sp3 construct on the BCAT-2 reporter as well as the inactivity of the small isoforms on both, the BCAT-2 and the SV40 promoter is dependent on the presence of the SUMO target lysine K551.

To this end, we introduced mutations that prevent SUMOylation (K551 mutations) into the full-length Sp3 construct from which only the two long isoforms are expressed, and into the Sp3Δ1.+2.AUG construct from which only the two small isoforms are expressed after transfection (Fig. 7A). Western blot analyses demonstrate that the small isoforms are expressed at higher level as compared to the long isoforms (Fig 7B).
Different to the wild-type long isoforms, the Sp3li-K551R mutant strongly activated the BCAT-2 reporter (Fig. 7C). Similarly, on the SV40 promoter, the Sp3li-K551R mutant is a 2-3fold stronger activator compared to the Sp3liWT (Fig. 7D). Like the wild-type, the SUMOylation-deficient small isoforms of Sp3 (si-Sp3K551D) were unable to activate BCAT-2 (Fig. 7C). However, the SV40 promoter that is not activated by the wild-type small isoforms becomes strongly activated by the Sp3si-K551D mutant (Fig. 7D). This result shows that also the inactivity of the small Sp3 isoforms that contain a single glutamine-rich activation domain is to some extent due to posttranslational modification by SUMO.

Subcellular localization of Sp3 isoforms—We wanted to know whether the apparent differences in regulatory properties of the small and long isoforms of Sp3 may reflect differences in their subcellular localization. Sp3-deficient KO MEFs and SL2 cells were transfected with appropriate expression constructs for the long and the small wild-type Sp3 isoforms, as well as with the corresponding SUMOylation-deficient mutants. Immunostainings with anti-Sp3 antibodies revealed that in both cell types the wild-type isoforms and the SUMOylation-deficient mutants were located in the nucleus exhibiting a sponge-like, diffuse appearance (Fig. 8). 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 localisation.
DISCUSSION

Four different isoforms of the Sp3 exist that derive from different translational initiation site—Twelve years after the original cloning of the transcription factor Sp3 (13,14), we unraveled the puzzling complex Sp3 antigen pattern observed in immunoblots. Sp3 is expressed in four different isoforms that differ in their N-terminal extension. In addition, all four isoforms are targets of posttranslational modification by SUMO, which accounts for the eight Western blot signals.

The four isoforms of Sp3 derive from four AUG translational start sites. At this stage, we can only speculate on the mechanisms governing different translational initiation within the Sp3 mRNA. The second AUG (GaaAUGG) and the fourth AUG (GcuAUGG) but not the neighboring first AUG (AcuAUGa) and the third AUG (AcaAUGa), respectively are embedded in an optimal context. Thus, leaky scanning at the first AUG leading to initiation at the second AUG, and leaky scanning at the third AUG leading to initiation at the fourth AUG is likely to occur. But how can initiation at the internal third AUG be explained? An internal ribosomal entry site might be present in the Sp3 coding region that recruits ribosomes internally although there is a strong debate whether cellular internal ribosome entry sites exists at all (24) (and Letters to the Editor, Mol. Cell. Biol. 21, p. 8238-8246). Secondary structure prediction using the Mfold web server (25) revealed striking stable RNA hairpins involving an extreme GC-rich Sp3 mRNA region between nucleotide +58 and +279 relative to the transcriptional start site. Possibly, some ribosomes stall at
these hairpins, shunt and subsequently reinitiate at the downstream third and fourth AUGs. Stable secondary structures might also explain, why simultaneous mutation of the first two AUG codons resulted in the appearances of a number of new long isoforms initiating at non-AUG start codons. Scanning ribosomes may stall at the hairpin structures thereby initiating at non-AUGs that are in an optimal context.

An upstream open reading frame is involved in regulating Sp3 isoform expression—Initiation site selection at the first and second AUG is dependent on a short 30-nucleotide uORF. Mutation of the uAUG codon leads to exclusive initiation at the first AUG. Thus, the uORF is essential for the synthesis of the second long Sp3 isoform. Another mutation that brings the uAUG in an optimal context leads to predominant initiation at the second AUG start codon. This result could be explained also by a leaky scanning and reinitiation model. The uAUG is in a suboptimal context. Thus, leaky scanning over the uAUG would lead to initiation at the first AUG. Other ribosomes translate the uORF and reach the terminator codon. According to current models, the 40S subunit may hold on to the mRNA, resume scanning and reinitiate at a downstream AUG (26). Reinitiation, however, is most efficient when the uORF terminates some distance before the next AUG to re-acquire Met-tRNAi-εIF-2 for downstream AUG recognition (26). Since the distance between the uORF stop codon and the next AUG is only 17 nucleotides, these ribosomes would preferentially use the second AUG. Such a scenario would explain why predominantly the second AUG was used, when the uAUG codon was placed in an optimal
Kozak sequence (more ribosomes initiate at the uAUG). In addition, this model explains also the shift towards initiation at the first AUG when the distance between the uORF stop codon and the first AUG was increased (insertion mutants described in Fig. 3).

Functional differences between individual isoforms-The most obvious difference between the two long isoforms and the two small isoforms of Sp3 is the presence of two and one single glutamine-rich domains, respectively. Both Q-rich domains can act as strong activation domains on their own when fused to a heterologous DNA-binding domain (8). Accordingly, the long isoforms containing two activation domains do have a stronger activation potential than the short isoforms. The SV40 promoter for instance becomes activated upon expression of the long Sp3 isoforms but not by the small isoforms that contain a single activation domain. However, in other promoter settings exemplified by BCAT-2 the long isoforms of Sp3 are also inactive. Thus far, we do not understand the different activation capacities of Sp3 on different promoter settings. Clearly, it is not simply the number of Sp3 binding sites nor is the spacing of the Sp3 binding sites decisive for the activation by the long isoforms. A promoter that contains only a single GC-box becomes also activated by the long Sp3 isoforms (8) (and data not shown). BCAT-2 derivatives with variable distances of the two GC-boxes can also not be activated by Sp3 isoforms (unpublished data). We speculate that different chromatin structures that become established on different promoter settings are responsible for the observed activation differences.
A major determinant of Sp3 activity is the posttranslational modification by SUMO. SUMOylation takes place exclusively at lysine K551 present in all four isoforms. Mutation of K551 strongly enhanced transcriptional activity of the long Sp3 isoforms. However, also the inactivity of the small isoforms depends on SUMO modification. The small Sp3 isoforms in which K551 was mutated strongly activated the SV40 promoter. Two additional SUMO acceptor motifs (ΨKXE) are present exclusively in the longest isoform (VK9QE) or in both long isoforms (IK120DE), respectively. Both sites, however, do not become SUMOylated neither in vivo nor in vitro.

At this stage we do not know the functional relevance of having two long Sp3 isoforms. Based on transfection results, their activation capacities are similar. This does not mean that their function in vivo on natural promoters is identical and that both isoforms occupy the same promoter at the same time. For instance, the transcription factor C/EBPβ exists in three isoforms. The two long isoforms of C/EBPβ, designated LAP* and LAP, differ in only 21 amino acids at the N-terminus. Both isoforms are strong activators on plasmid-based gene transcription. Nevertheless, LAP* and LAP are strikingly different in their ability to activate chromatin-embedded chromosomal genes because the extreme N-terminus present exclusively in LAP* recruits the SWI/SNF complex involved in chromatin remodeling (27). Sequence comparison of the N-terminus of LAP* with the N-terminus of Sp3 revealed no obvious similarities. It remains to be established whether the two long isoforms of Sp3 exhibits distinct activities on chromatin-embedded endogenous genes. Generating knockin mice that
express only single isoforms of Sp3 might also help to clarify the function of individual isoforms.

*Is Sp3 isoform expression altered under certain conditions?* - Sp3 isoform expression is to some extent reminiscent of the isoform expression described for the transcription factors C/EBPα and C/EBPβ (28) as well as of the transcription factor SCL (29). In all three cases isoforms arise from unique mRNAs by differential initiation of translation. Like Sp3 isoforms, C/EBP isoforms retain different parts of the amino terminus and display different functions in gene regulation. Moreover, upstream open reading frames regulate initiation from different translation initiation sites. In the case of C/EBPα and β deregulated translational control interfered with terminal differentiation and induced cell transformation in 3T3-L1 adipocytes (28). Thus far, we do not know whether and how isoform expression of Sp3 is regulated *in vivo*. Serum removal or serum addition did not change the relative ratio of the different isoforms (unpublished data) making it unlikely that isoform expression changes during the cell cycle.

Initiation of translation is affected by a number of pathways that control the activity and level of eukaryotic translation initiation factors (eIFs) (30). For instance, at high eIF-2 and eIF-4E activity comparatively more truncated C/EBP isoforms are expressed, whereas at lower activity of these factors expression of long isoforms dominates (28). We have transfected full-length Sp3 along with expression constructs for the eukaryotic initiation factors eIF2 and eIF4E and subsequently analyzed Sp3
isoform expression. Under our experimental conditions, the isoform ratio did not change. Moreover, we grew cells in the presence or absence of rapamycin and 2-aminopurin. Rapamycin diminishes the activity of eIF4E, 2-aminopurin enhances the activity of eIF2α. Again, we could not detect a change in Sp3 isoform expression (data not shown). Thus at this stage, we do not know under which physiological conditions, Sp3 isoform ratio alterations may take place. A significant shift towards the long isoforms of Sp3, however, is observed in Sp1-/- ES cells (unpublished data) 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.

Sp1 versus Sp3-The structural similarity between Sp1 and Sp3 as well as their ubiquitous expression suggested originally that they have similar properties and exert similar functions. Detailed biochemical and biological studies, however, highlighted significant differences, including different knockout phenotypes, different posttranslational modifications and expression of different isoforms (Fig. 9). These studies establish Sp3 as a transcription factor with properties considerably distinct from Sp1. Most obviously, Sp3 is expressed as four isoforms that all can become posttranslationally modified by a SUMO moiety. In addition unlike Sp1, Sp3 is not glycosylated. Moreover, Sp1 is highly phosphorylated. Whether any of the isoforms of Sp3 can be a substrate for phosphorylation under certain conditions remains to be established. A modification that has been described for both molecules is acetylation. Acetylation of Sp1 likely
occurs at lysine residues within the DNA-binding domain (31), while the location of acetylation of Sp3 has not yet been precisely determined. Previously, we have shown that mutation of the SUMO lysine K551 impaired acetylation of Sp3 in vivo (32). This finding suggested originally that the same lysine residue that is a target for SUMOylation is also a target for acetylation. However, 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 (unpublished data). These results suggest that it may not be K551 that becomes acetylated in vivo. We rather speculate that SUMO-modified Sp3 might recruit acetyl transferases that in turn acetylate other lysine residues within the Sp3 protein.

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REFERENCES

1. Philipsen, S., and Suske, G. (1999) *Nucleic Acids Res.* **27**, 2991-3000
2. Suske, G. (1999) *Gene* **238**, 291-300
3. Marin, M., Karis, A., Visser, P., Grosveld, F., and Philipsen, S. (1997) *Cell* **89**, 619-628
4. Bouwman, P., Göllner, H., Elsässer, H. P., Eckhoff, G., Karis, A., Grosveld, F., Philipsen, S., and Suske, G. (2000) *EMBO J.* **19**, 655-661
5. Göllner, H., Dani, C., Phillips, B., Philipsen, S., and Suske, G. (2001) *Mech. Dev.* **106**, 77-83.
6. Bouwman, P., and Philipsen, S. (2002) *Mol. Cell. Endocrinol.* **195**, 27-38
7. Hagen, G., Müller, S., Beato, M., and Suske, G. (1994) *EMBO J.* **13**, 3843-3851
8. Dennig, J., Beato, M., and Suske, G. (1996) *EMBO J.* **15**, 5659-5667
9. Ross, S., Best, J. L., Zon, L. I., and Gill, G. (2002) *Mol. Cell* **10**, 831-842
10. Sapetschnig, A., Rischitor, G., Braun, H., Doll, A., Schergaut, M., Melchior, F., and Suske, G. (2002) *EMBO J.* **21**, 5206-5215
11. Kennett, S. B., Udvadia, A. J., and Horowitz, J. M. (1997) *Nucleic Acids Res.* **25**, 3110-3117
12. Oleksiak, M. F., and Crawford, D. L. (2002) *Mol. Biol. Evol.* **19**, 2026-2029
13. Hagen, G., Müller, S., Beato, M., and Suske, G. (1992) *Nucleic Acids Res.* **20**, 5519-5525
14. Kingsley, C., and Winoto, A. (1992) *Mol. Cell. Biol.* **12**, 4251-4261
15. Suske, G. (2000) *Methods Mol. Biol.* **130**, 175-187
16. Lange, S., Auerbach, D., McLoughlin, P., Perriard, E., Schafer, B. W., Perriard, J. C., and Ehler, E. (2002) *J. Cell Sci.* **115**, 4925-4936
17. Brasier, A. R., and Fortin, J. J. (1997) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol.
18. Hall, C. V., Jacob, P. E., Ringold, G. M., and Lee, F. (1983) *J. Mol. Appl. Genet.* **2**, 101-109
19. Kolell, K. J., and Crawford, D. L. (2002) *Mol. Biol. Evol.* **19**, 216-222
20. Morris, D. R., and Geballe, A. P. (2000) *Mol. Cell. Biol.* **20**, 8635-8642
21. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001) *J. Biol. Chem.* **276**, 12654-12659.
22. Jackson, S. P., and Tjian, R. (1988) *Cell* **55**, 125-133
23. Jackson, S. P., and Tjian, R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1781-1785
24. Kozak, M. (2001) *Mol. Cell. Biol.* **21**, 1899-1907
25. Zuker, M. (2003) *Nucleic Acids Res.* **31**, 3406-3415
26. Kozak, M. (1999) *Gene* **234**, 187-208
27. Kowenz-Leutz, E., and Leutz, A. (1999) *Mol. Cell* **4**, 735-743
28. Calkhoven, C. F., Muller, C., and Leutz, A. (2000) *Genes Dev.* **14**, 1920-1932
29. Calkhoven, C. F., Muller, C., Martin, R., Krosl, G., Pietsch, H., Hoang, T., and Leutz, A. (2003) *Genes Dev.* **17**, 959-964
30. Hershey, J. W. (1991) *Annu. Rev. Biochem.* **60**, 717-755
31. Suzuki, T., Kimura, A., Nagai, R., and Horikoshi, M. (2000) *Genes Cells* **5**, 29-41
32. Braun, H., Koop, R., Ertmer, A., Nacht, S., and Suske, G. (2001) *Nucleic Acids Res.* **29**, 4994-5000
FIGURE LEGENDS

FIG 1. **Complexity of endogenous Sp3 protein expression.** A, 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 in RIPA buffer (lane 5) and proteins separated through 6% SDS polyacrylamide gels. After blotting, PVDF membranes were incubated with Sp1- or Sp3-specific antibodies as indicated. B, *In vitro* transcribed/translated Sp3 proteins radiolabeled with ^35S were subjected to *in vitro* SUMOylation reactions using purified recombinant E1 enzyme (Aos1/Uba2), Ubc9 and PIAS1 (10) in the presence (lane 1, +) or absence (lane 2, -) of recombinant SUMO-1. Reaction products were separated through 5% SDS-PAGE and visualized by autoradiography. 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 (see Figure 2). For Sp1, the asterisk indicates the phosphorylated form.

FIG 2. **The four isoforms of Sp3 derive from four in frame AUG codons.** A, schematic drawing of Sp3 cDNA eg. RNA mutants cloned in a vector appropriate for *in vitro* transcription/translation (T7- and Sp6-promoter-containing pSPT18 vector), transient expression in SL2 cells (*Drosophila melanogaster* actin promoter-driven pPac vectors) and transient expression in mammalian cells (CMV-driven C-terminal HA-
epitope vectors). Mutant codons are depicted in italic letters. B, coupled in vivo transcription/translation reactions. Sp3 wild-type cDNA and mutants were in vivo transcribed/translated. Subsequently, 35S-radiolabeled proteins were separated through 5% SDS-PAGE and visualized by autoradiography. C, schematic drawing of the Sp3 domain structure and the four isoforms. The two long isoforms of Sp3 (Sp3li-1 and Sp3li-2) contain both two glutamine-rich activation domains (AD, indicated in grey); the two small isoforms contain only one activation domain. M depicts the four methionine residues. The DNA-binding domain (DBD) consists of three zinc fingers indicated as black boxes. K551 indicates the lysine residue that is targeted by SUMO. D, Western blot analyses of transient transfected 293 cells. Cells were transfected with 3 µg of expression plasmids for C-terminally HA-tagged Sp3 mutants as indicated. Cells were lysed in SDS-containing buffer, separated through 6% SDS PAGE, and Sp3 isoform expression analyzed by Western blot using anti-HA antibodies. To separate the two long isoforms, an independent long gel run was performed (left). E, Western blot analyses of transiently transfected SL2 cells. Cells were transfected with 1 µg of expression plasmids for Sp3 mutants as indicated. Nuclear extracts were prepared and 10 µg of protein extract was analyzed by Western blotting using anti-Sp3 antibodies.

FIG. 3. An upstream ORF regulates expression of the Sp3 long isoforms. A, sequence of the 5’-non-translated region of the Sp3 mRNA. The uORF is boxed, the first start codon highlighted in grey. B, schematic drawing of uORF mutants. C, and D, Western blot analyses of transfected
SL2 (C) and 293 (D) cells. Cells were transfected with 1 µg of expression plasmids for Sp3WT, Sp3uAUGm, Sp3uAUGoK, Sp3In-1, Sp3In-2 or Sp3PolyG mutants as indicated. Ten µg of nuclear extract (SL2 cells) or SDS extracts (293 cells), respectively were separated through 6% SDS polyacrylamide gels and analyzed by Western blotting using anti-Sp3 (C) or anti-HA (D and E) antibodies.

FIG. 4. Posttranslational modification of 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 HA-Sp3K551R), C-terminal HA-tagged (Sp3WT-HA and Sp3K551R-HA) or untagged Sp3 (Sp3WT and Sp3K551R) 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, In vitro SUMOylation of Sp3WT, Sp3K551R and Sp3Δ1AUG (see Fig. 2) proteins. In vitro transcribed/translated 35S-labeled Sp3 proteins were subjected to in vitro SUMOylation reactions (10) using recombinant E1 enzyme, Ubc9 and PIAS1 in the presence (+) and absence (-) of SUMO-1. Reaction products were separated by SDS-PAGE and visualized by autoradiography. Molecular weight markers (M) are also shown.
FIG. 5. **Unlike Sp1, Sp3 is not posttranslationally modified by glycosylation.** Whole SDS cell extracts (CE) prepared from 293 cells or transfected SL2 cells were subjected to wheat germ agglutinin (WGA) affinity chromatography and subsequently analyzed by Western blotting using anti-Sp1 or anti-Sp3 antibodies as indicated. In lanes 1, 3, 5, 6, 9, 11, 13 and 15 100 µg of whole cell extract protein were separated. In lanes 2, 4, 6, 8, 10, 12, 14 and 16 wheat germ agglutinin bound material was analyzed. In lanes 4 and 8 100 mM N-acetyl D-glucosamine (GlcNac) was included in the WGA binding reaction.

FIG. 6. **Functional analyses of Sp3 isoforms.** A, DNA binding capacity of Sp3 mutant proteins in SL2 cells. Gel retardation assays were performed with crude nuclear extracts from SL2 cells transfected with 1 µg of expression plasmids for Sp3 isoform mutants as indicated. All reactions contained 0.2 ng of 32P-labeled GC-box oligonucleotide and 5 µg of protein extract. B, SL2 cells were transfected with 4 µg of the BCAT-2 reporter plasmid or the SV40 promoter driven luciferase reporter plasmid pGL3 along with 0.5 µg or 2 µg of expression plasmids for wild-type Sp3 or Sp3 mutants as indicated. The fold activation values represent mean values of at least two independent transfections.

FIG. 7. **Point mutations that prevent SUMO modification strongly enhance Sp3 activation capacity.** A, Schematic drawing of Sp3 long (Sp3li) and small (Sp3si) isoforms. The glutamine-rich activation domains (AD), the SUMOylation site (IKEE) and the DNA-binding domain (DBD)
are indicated. Mutations that replace lysine 551 were introduced in expression constructs for wild-type Sp3 and the Δ1.2.AUGm mutant. B, Transient expression of Sp3 long and short isoforms and lysine K551 mutants in SL2 cells. Western blots analyses was performed with whole cell extracts (SDS lysis) from SL2 cells transfected with 1 µg expression plasmid for the wild-type Sp3 short isoforms (si-WT, lane 2), the Sp3K551D short isoform mutant (si-K551D, lane 3), wild-type long isoforms (li-WT, lane 4) and Sp3K551R long isoform mutant (li-K551R, lane 5). The arrows point to the SUMO-modified short and long WT Sp3 isoforms (lanes 2 and 4). C, and D, SL2 cells were transfected with 20 ng and 500 ng of expression plasmids for the long (Sp3li) and short (Sp3si) Sp3 isoforms and corresponding lysine 551 mutants along with 4 µg of the BCAT-2 plasmid (C) or 4 µg of the SV40 promoter driven luciferase reporter plasmid pGL3 (D).

FIG. 8. Subcellular localisation of Sp3 isoforms and corresponding SUMOylation-deficient mutants in MEFs and SL2 cells. Sp3 was detected by immunostaining with a rabbit anti-Sp3 antibody and a FITC-(in A) or AF594-conjugated (in B) secondary antibody. Control stainings of nuclei were performed with DAPI. 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-K551R and Sp3si-SD) are shown. The Sp3siSD mutants contains 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 1 µg of expression constructs for Sp3 isoforms and mutants as indicated.

FIG. 9. Comparison of known structural, biological and biochemical features of the transcription factors Sp1 and Sp3 (see discussion chapter for details).
Figure 1

A

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| ES-Sp3/− | ES-Sp3/− | ES-WT | 293 cells | RIPA/SDS | Ishikawa |
| Sp1+/− | Sp1 | Sp3li-SUMO | Sp3li-1 | Sp3li-2 | Sp3si-SUMO |
| Sp1 | Sp3 | Sp3si-3 | Sp3si-2 | Sp3si-4 |

B

In vitro tx/tl

1 +

2 −
Figure 2

A

B

C

D

E

Figure 2

A

B

C

D

E
Figure 3

A

ggcuccaccuuuuguguuuccgccacagucuauc
aaaaauaggaaaaaaaauccccggaccgcucgccg
cgugucgccgccgccucuagaaucaccuccucccg
cgccgccccucuccuccucuccaccauguguaagg
cggcgggagccccggcugaggugcccuaaaccac
acu AUG ACC GCU CCC GAA . . .

B

|    | uAUG | 1. AUG | WT |
|----|------|--------|----|
|    | uUAG | 1. AUG | uAUGm |
| ccaccuAUGg | 1. AUG | uAUGoK |
| uAUG | 1. AUG | PolyG |
| uAUG | 1. AUG | In-1 |
| uAUG | 1. AUG | In-2 |

C

D

Mock WT uAUGm uAUGok

WT In-1 In-2 PolyG

Sp3li-1 Sp3li-2

Sp3li-1 Sp3li-2

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Figure 4

A

MTAPEKVQEM...

Consensus

ΨKXE

B

Sp3li-1

Sp3li-2

Sp3li-SUMO

Sp3ii

C

Sp3A1.AUG

Sp3WT

Sp3K551R

M - + - + - +

Sp3li-1+2-SUMO

Sp3li-1

Sp3li-2

Sp3si-3-SUMO

Sp3si-4-SUMO

Sp3si-3

Sp3si-4

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Figure 5

293 cells (endogenous)

| CE | WGA | CE | GlcNAC |
|----|-----|----|--------|
| 1  | 2   | 3  | 4      |
| Sp1|     |    |        |

| CE | WGA | CE | GlcNAC |
|----|-----|----|--------|
| 5  | 6   | 7  | 8      |
| Sp3|     |    |        |

SL2 cells (transfected)

| CE | WGA | CE | WGA | CE | WGA | CE | WGA |
|----|-----|----|-----|----|-----|----|-----|
| 9  | 10  | 11 | 12  | 13 | 14  | 15 | 16  |
| Mock| Sp3K551R| Sp3WT| Sp3li-SUMO | Sp3li | Sp3si | Sp1 |
Figure 6
Figure 7

A

Sp3li

AD

AD

ID

DBD

Sp3si

IKEE

B

Mock

si-WT

si-K551D

ii-WT

ii-K551R

C

BCAT-2

2xGC

45

20 500

1.0 1.2

WT

K551R

Sp3li

20 500

1.1 1.0

WT

K551D

Sp3si

D

SV40

5xGC

57

20 500

1.7 3.8

WT

K551R

Sp3li

20 500

1.1 1.6

WT

K551D

Sp3si
Figure 8

A

WT MEFs  KO MEFs  KO MEFs + Sp3li-WT  KO MEFs + Sp3li-K551R  KO MEFs + Sp3si-WT  KO MEFs + Sp3si-SD

αSp3  αSp3  αSp3  αSp3  αSp3  αSp3

DAPI  DAPI  DAPI  DAPI  DAPI  DAPI

B

SL2 + Sp3li-WT  SL2 + Sp3li-K551R  SL2 + Sp3si-WT  SL2 + Sp3si-K551D

αSp3  αSp3  αSp3  αSp3

DAPI  DAPI  DAPI  DAPI
Figure 9

| KO phenotype | Embryonic lethal (E10) | Neonatal lethal |
|--------------|-----------------------|----------------|
| Transcriptional activity | ++ | +/- |
| Posttranslational modifications | | |
| Phosphorylation | + | ? |
| Glycosylation | + | - |
| Acetylation | + | + |
| SUMOylation | - | + |
| Isoforms | 1 | 4 |
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