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Structure and functional characterization of single-strand DNA binding protein SSDP1: Carboxyl-terminal of SSDP1 has transcription activity

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Abstract

LIM-homeodomain transcription factors control a wide range of developmental processes, such as early patterning of the embryonic development, organ formations of brain, limbs, and eyes. Molecular mechanisms of the underlying processes involve complicated multiple protein complexes that direct transcription activation of target genes, protein-protein interactions, and transcriptional regulations. Among those molecules, cofactor Ldb1, interacting with LIM/homeobox family transcription factor, defines a tetrameric protein complex in controlling downstream genes of transcriptional regulation. In addition, SSDP specifically involves this complex supported by showing that both SSDP1 and SSDP2 bind to Ldb1 in vivo. Here it has been found that SSDP1 itself is a transcription factor that has transcription activity independently. Furthermore, C-terminal of SSDP1 possessing an entire transcription activity in vivo, confirmed in both yeast and mammalian cells, has been defined. Interestingly, the transcriptional function of SSDP1 was not required for the interaction with Ldb1. Thus, biochemical data of SSDP1 presented by this study provides biochemical evidence for a better understanding of transcriptional regulation.

Keywords: SSDP1; Transcription factor; Nuclear localization

Transcriptional regulation is a key event for controlling developmental programs in a variety of species. Among these, transcription factors play essential roles for conducting organ patterning and development, and cell differentiation. Extensive studies have demonstrated that the members of the LIM-homeodomain transcription factors are involved in a variety of developmental processes [1]. Transcription co-factor Ldb1, which was the first to be identified as a LIM domain interacting protein, is also involved in these processes [2,3]. Cumulative evidence shows that, LDB1, in addition to its association with the LIM/homeobox (Lhx) family, involves other multiple protein complexes, such as LIM only, GATA and Otx transcription factors. Hence, Ldb1 knockout mice die very early in embryonic development and have several defects in organ patterning, such as heart anlage and brain truncation [4], suggesting that Ldb1 plays an essential requirement for embryonic development. Ldb1 functions to be believed to form a tetrametric complex for conducting DNA binding and transcriptional regulation. However, the detailed biochemical feature of Ldb1 containing protein complexes is not completely known.

Genetic evidence shows that single-strand DNA binding protein SSDP family is conserved protein among various species and its functions are essential for embryonic development. Recent data show that Ldb1 interacts with single-strand DNA binding protein SSDP family in HeLa cell nuclear extraction in both immunoprecipitation [5] and yeast two-hybrid systems [6]. Further, the domain mapping experiment shows that the middle conserved domain

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(LCCD) of the Ldb1 specifically interacts with the N-terminal of SSDP2 [6]. The SSDP family is conserved protein among various species and its functions are essential for embryonic development because SSDP2 knockout is lethal in mice [6]. Similarly, SSDP null mutants in *Drosophila* are cell-lethal [6]. Transgenic study demonstrates that in *Xenopus*, mouse SSDP1 mRNA induces axis induction with Lim1 and Ldb1 coactivation. A recent report shows that reduced expression of SSDP1 in mice leads to head shrinking phenotype [7], indicating that SSDP1 specifically involves head development of mouse embryos. Taken together, it suggests that SSDP and its forming complexes may provide an essential role in molecular regulation of gene transcription in development.

Although various studies have illustrated functions of the SSDP family in embryonic development, the biochemical nature of SSDP is largely unknown. I have performed a yeast two-hybrid screening, revealing that full-length SSDP1 turned out to cause reporter gene activation, suggesting that SSDP1 has transcription activity. Here, I describe that SSDP1 is a transcription factor, whose function is independent of Ldb1 in both yeast and mammalian cells. Further study clearly demonstrates that C-terminal of SSDP1 possesses total transcription activity.

Results

The features of the SSDP1 domain structure and multiple alignment

Bioinformatics provides a comprehensive approach for analyzing virtual domain structures within multiple molecules by sequential alignments. ProDom is a comprehensive set of protein domain families automatically generated from the SWISS-PROT and TrEMBL sequence databases. To dissect mouse SSDP1 domain structures, I performed scan mouse SSDP1 with a 361 amino acid sequence that searched the Prodom program (http://www.prodom.com) for identifying protein decomposition, revealing mouse SSDP1 domains based on the concensus sequence of multiple proteins. As shown in Fig. 1, the Prodom program demonstrated that SSDP1 had four major domains, assigned as D1, D2, D3, and D4 (Fig. 1).

SSDP1 domain 1 contained from amino acid 1 to amino acid 92. The consensus sequence of this domain of SSDP1 was MFAKGKGSAVPSDGQAREKLALYVYEYLLHV GAQKSAQTFLSEIRWEKNITLGEPPGFLHSWWCV FWDLYCAAPERRDTCEHSSEAKAFHDY. Multiple alignments indicated that this domain was highly conserved, identified in several species from *Caenorhabditis elegans, Xenopus*, rat, mouse, and human (Fig. 1A). SSDP1 domain 1 was found to be the closest domain in 19 members among the above species. This D1 domain of SSDP1 was equivalent to LUFS domain identified previously, whose function was believed to mediate the interaction between SSDP1 and Ldb1 protein. Domain mapping displays that the function of SSDP1 involves Lhx family member protein complexes, mediated through LDB-1 binding, a transcription regulator [6]. The second domain (D2) of SSDP1 was the closest domain identified among 20 members of the family, whose protein sequence was SPRYA GGPRPPIRMGNQPPGGVPGTQPLLPNSMDPTRQQ GHPNMGGS. It was of 54 amino acids in length (Fig. 1B). The function of this domain of SSDP1 was unknown. The third domain of SSDP1 was assigned as domain 3 because it contained from amino acid 192 to amino acid 220. No further function for this domain has been assigned so far. Interestingly, this domain was rich in proline amino acid that might mediate the complicated complex formation of nuclear proteins.

Finally, domain 4 of SSDP1 was the closest domain among 19 members in the family, whose length was 104 amino acids long from amino acid 258 to amino acid 361. It had ADSTNSSDNIYTMINPVPPGGSRSNFPM GPGSDGPMGGMGGMEPHHMNGSLGSGDIDGLP KNSPNNISGISNPPGTPRDDGELGGNFLHSFQND NYSPSMTMSV. This domain of SSDP1 has not been characterized yet.

SSDP1 activates LacZ gene detected by β-galactose assay in yeast cells

To identify the interacting partner for SSDP1, I had designed full-length cDNA fused with Gal4 DNA binding domain under control expression of yeast promoter to ensure the fusion gene expression in yeast cells (Fig. 2A). Sequential transformation was performed and then the mouse brain cDNA library was screened in four dropout plates (-Trp, -Leu, -His, -Ade), with 3-AT 15 mM concentration. Surprisingly, all transformed yeast cells grew in the highly stringent selection plates in three days of incubation (Fig. 2B). Obviously, one of the most possible reasons for this occurance could be that SSDP1 had autotranscriptional activity, leading to activating the selective genes, such as histidine and adenine, in yeast cells, without requiring Gal4 activation domain in protein-protein interaction.

To prove this hypothesis, I believed that SSDP1 itself in the bait selection plates was able to activate the reporter gene, such as LacZ gene, without requiring Gal4 activation domain in prey vector. In order to complete this experiment, yeast cells were transformed with Gal4-BD-SSDP1 and selected in negative tryptophane plates (-Trp). Yeast cell clones were picked up and subjected to β-galactose analysis. The results, I expected, showed that LacZ expression of transformed yeast cells harboring wild type SSDP1, but not vector, was detectable in comparison to vector alone (Fig. 2D). Thus, SSDP1 alone acted as a transcription factor in yeast, whose function was independent of Ldb1 expression.

Obviously, the next step was to identify which specific domain of SSDP1 was responsible for transcriptional activation by domain mapping analysis. According to domain structure of SSDP1 as described above, each domain of SSDP1 was inserted to the C-terminal of GAL4-DNA

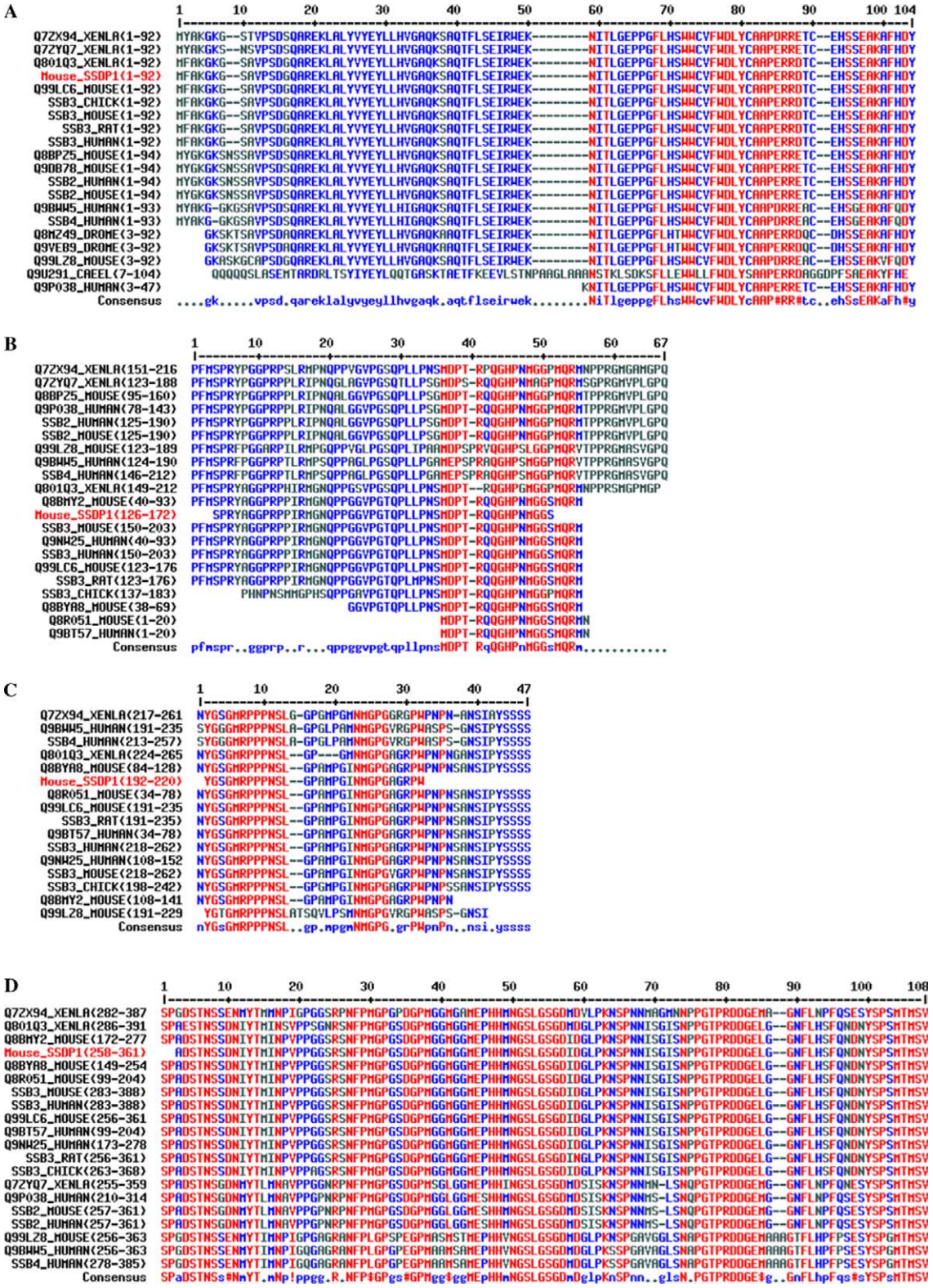

Fig. 1. Semantics of SSDP1 domains and multiple alignments. 361 amino acid full-length SSDP1 was analyzed in Prodom web-based program, domain decomposition software for free use. The significance of multiple alignments indicates that SSDP1 can be separated into four individual domains. Blue color represents from bacterial and green color means from viral. Red color stands for archaeal. (A) The first motif is the graphic representation in the family. Nineteen family members have a motif representation, consistent throughout the whole database. This 1–92 amino acid motif is equivalent to LUFS domain as identified before. (B) The second domain of mSSDP1 is highly conserved from chicken to humans. The significant feature of this domain is as of proline-rich. Third domain has not been assigned any specific function in being yet. (C) This domain in mSSDP1 is 28 amino acids, a shortest domain. The motif GMRPPPNSL is obviously natural. No specific function is related to this domain. (D) C-Terminal domain of SSDP1 consists of 102 amino acids in mSSDP1. Its function has not been characterized yet. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

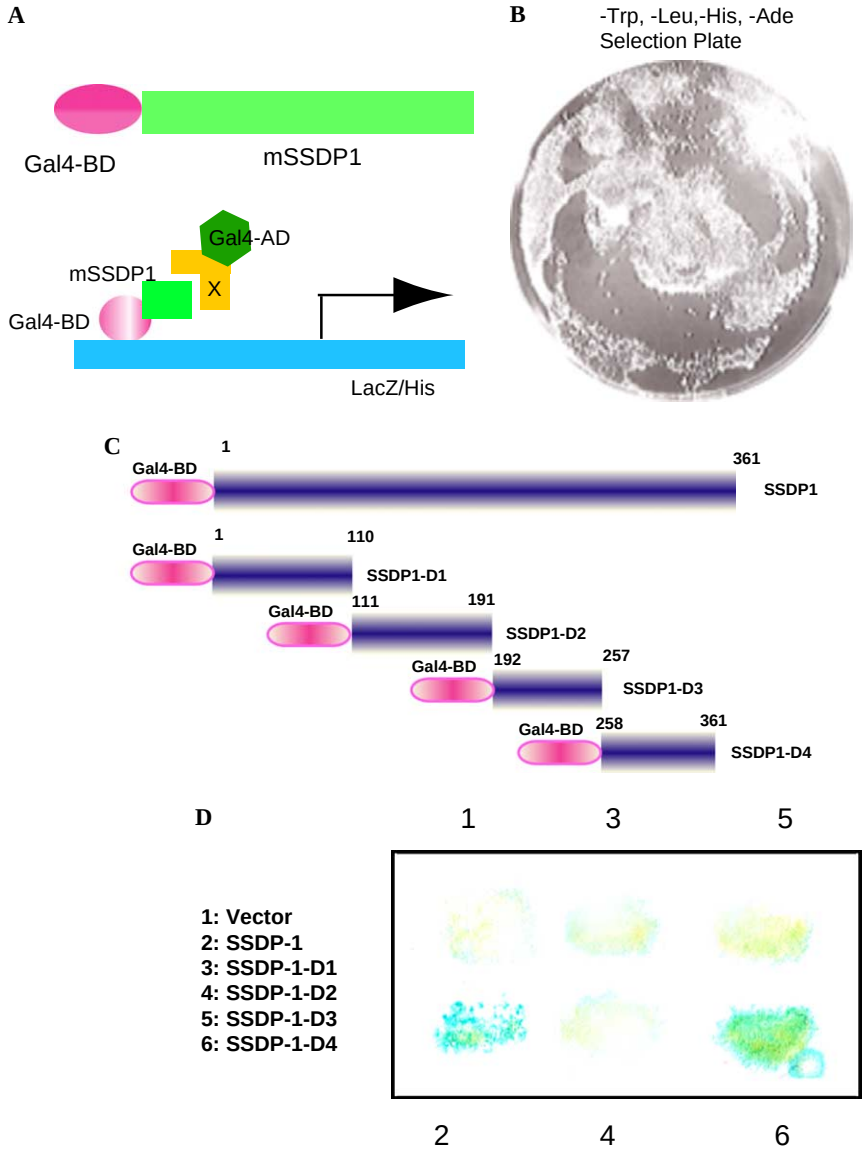

Fig. 2. Mouse SSDP1 is the transcription factor in yeast cells. (A) Simple schematic representation of Gal4-DNA binding domain fusion with mSSDP1 for yeast cell transformation. Mouse SSDP1 was tagged Gal-BD in its N-terminal. The principle of yeast two-hybrid system was presented and the reporter gene LacZ and histidine genes are activated when the bait and prey interact in yeast cells. (B) A typical example of a four dropout (-Trp, -Leu, -His, -Ade) selective plate is shown here when full-length mSSDP1 was used for yeast two hybrid screening. All transformed yeast cells were grown under high selection condition, with 15 mM 3-AT in the plates. (C) Schematic demonstration of the SSDP1 and its truncated versions are fused with Gal4-DBD. (D) SSDP1 and C-terminal of SSDP1 have transcription activities. Wild-type SSDP1 and its truncated versions were individually transformed in AH109 yeast strains and the yeast cells were selected in -Trp plates, without the prey co-transformation. The yeast colons were picked up for β-galactose assay. Turning blue color within 30 min was considered a positive clone.

binding domain in-frame (Fig. 2C). To further fulfill the nature of the domains of SSDP1, the yeast cells were transformed and selected in -Trp selection plate. The yeast clones were picked up for LacZ gene expression by β-galactose assay. Data showed that, compared with that in yeast with vector control, LacZ expression was not significantly detectable in either SSDP1-D1, or SSDP1-D2, or SSDP1-D3 transformed cells (Fig. 2D). However, LacZ protein was significantly expressed in the C-terminus of SSDP1 transformed cells (Fig. 2D). It indicated that C-terminal of SSDP1 had high transcription activity comparable to that in full-length SSDP1 in yeast cells. Conclusively, the C-terminal of the SSDP1 contained transcription activity.

SSDP1 is a transcription factor in mammalian cells

In order to show transcription activity of the SSDP1 in mammalian cells, I constructed SSDP1 and its truncated versions in fusion with Gal4 DNA binding domain under CMV promoter, a strong promoter in mammalian cells, for controlling gene expression (Fig. 3A). Reporter gene luciferase was controlled by five times repeats of TATAA

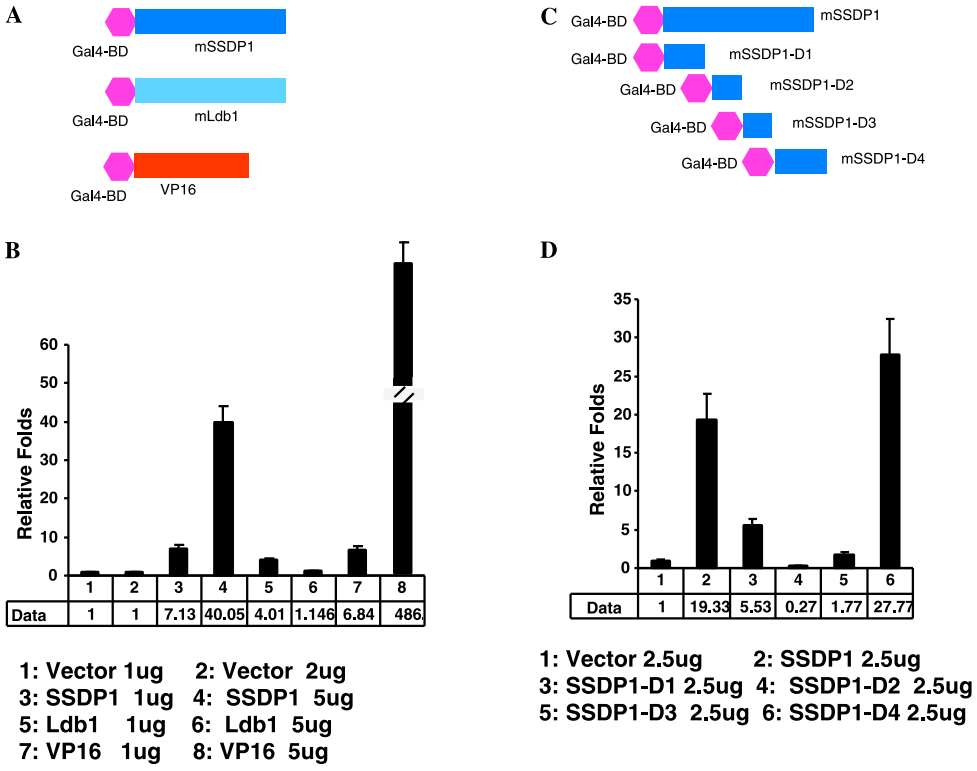

Fig. 3. Mouse SSDP1 and C-terminal SSDP1 possess transcription activity detected by reporter luciferase assay in vivo. (A) Schematic representation of mouse SSDP1, Ldb1, and VP16 fused with Gal4-DNA binding domain. CMV promoter controls fusion gene expressions of the GAL4-BD-SSDP1, Gal4-BD-Ldb1, and Gal4-BD-VP16, respectively. Ldb1 was used as a negative control while virus VP16, the strongest transcription factor, serves as a positive control. Gal4 DNA binding domain will bind to five repeats of the TATAA box located in the upper stream promoter of luciferase gene activation [8]. (B) SSDP1, not Ldb1, has transcription activity. Viral VP16 protein is the strongest transcription factor, serving as a positive control. Mouse SSDp1 and Ldb1 in two dosages (1 and 5 μg) were transfected with pG5E1B-Luc (2.5 μg) reporter plasmid and pTK-Rluc (1 μg) control plasmid in 293-T cells. The clear cell lysates were measured for luciferase activity. Relative folds of induction, compared to vector alone, were used for graphic representation in Microsoft Excel program. (C) Schematics of the mouse SSDp1 and its individual domains. Mouse SSDP1 and its truncated domains were individually fused to Gal4 DNA binding domain and hybrid proteins were inserted into expression vector of CMV promoter. (D) Only C-terminal of mSSDP1 has transcription activity. 293-T cells were transfected with either vector, or full-length SSDP1, or its truncated domains, respectively, combining with pG5E1B-Luc reporter plasmid and pTK-Rluc as a control. The total cell lysates were subjected to measure the luciferase assay. The relative folds of the induction were used for graphic presentation.

box for GAL4 DNA sequence [8]. SSDP1 and its truncated domains were cotransfected with p5G-Luc and pTK-RL in 293T cells and cell extracts were cleared by high-speed centrifugation. The total cell lysates were subjected to measurement of luciferase activity using dual-luciferase assay. The luciferase activity was measured and digitally normalized with empty vector pTK-RL as a control. Meantime, the Ldb-1, the closed binding partner of SSDP1, proved to no transcription activity in yeast two-hybrid screen (my unpublished data). However, virus VP16 had been proved to be the strongest transcription factor serving as a positive standard control in comparison. To implement this experiment, Gal4 DNA binding domain was fused with full-length SSDP1, Ldb-1, and VP16, respectively (Fig. 3A). First, I wanted to demonstrate that SSDP1, Ldb1, and VP16 acted in a dosage dependent manner. 293-T cells were transfected with mixed plasmids with an indicated amount (1 and 5 μg, respectively). The empty vector pTK-*Renilla* luciferase served as an internal control of normalizing the transfection efficiency. The luciferase activities were corrected by relative folds of inducing luciferase activity with control group as one unit. Gal4-VP16 was known to be the strongest transcription factor so far. The samples were assessed for luciferase activity after 48 h of cell transfection. Data showed that Ldb-1 did not increase luciferase activity in both 1 and 5 μg concentrations. Obviously, VP16 1 μg induced luciferase activity 7-fold while VP16 5 μg increased 486-fold of luciferase transcription activity. Interestingly, 1 μg SSDP1 transfectants induced 7-fold of the amount of the basal level of luciferase activity in vector control while 5 μg SSDP1 induced 40-fold of luciferase activity (Fig. 3B). In conclusion, these data indicated that SSDP1 was a transcription factor, whose function represented transcription activation.

To further analyze which specific domain of SSDP1 possessed the transcription activity, four domains of SSDP1 were individually fused with Gal4 DNA binding domain at its N-terminals and subsequently cloned into CMV promoter controlling gene expression (Fig. 3C). 5 μg SSDP1 full-length and its unique domains were cotransfected with

reporter gene for monitoring luciferase activity. The normalized data showed that, compared to vector alone, domain 1 of SSDP1 had not induced reporter luciferase activity (Fig. 3D). Domain 2 and domain 3 of SSDP1 induced basal levels of transcription activities, such as 1.5-fold and 1.8-fold of reporter luciferase activities, respectively. Surprisingly, domain 4 of SSDP1 induced 33-fold of luciferase activity. These data further indicated that carboxyl terminal of the SSDP1 had transcription activity in vivo (Fig. 4).

Single-strand DNA binding protein SSDP1 localizes in nuclear

To overcome the lack of SSDP1 antibody for indirect immunofluorescence staining assay, green fluorescent protein (GFP) was designed to investigate the protein's subcellular localization. To this end, full-length and truncated domains of SSDP1 were fused in-frame with the C-terminus of GFP. 293-T cells were transfected with GFP-SSDP1 and its domain fragments of SSDP1 for 48 h and the transfected cells were fixed and their images taken under an immunofluorescent microscope. In comparison to GFP alone, wild type SSDP1 exclusively located in nuclear with even distribution, showing perfect co-localization with nuclear dye marker stained with DAPI. Additionally, GFP-SSDP1-D1 was mainly stained in nuclear, while some of them expressed in cytoplasm. GFP-SSDP1-D2 was a large amount of protein stained in cytoplasm. Both GFP-SSDP1-D3 and GFP-SSDP1-D4 were found in cytoplasm. Accordingly, SSDP1 localizes in nuclear.

Discussion

In this paper, I have described the biochemical aspect of nuclear protein SSDP1 assessed through a variety of approaches. Detailed functional studies *in vivo* have revealed that single-strand DNA binding protein SSDP1 is a transcription factor in yeast and mammalian cells. Domain mapping experiment has demonstrated that C-terminal of SSDP1 possesses the entire transcription activity. Moreover, the function of SSDP1 transcription activity is not required for Ldb1, the close binding partner, as shown in yeast and mammalian cells. To my knowledge, the significance of this paper is the first biochemical and functional evaluation of a SSDP1 protein, giving us specific and

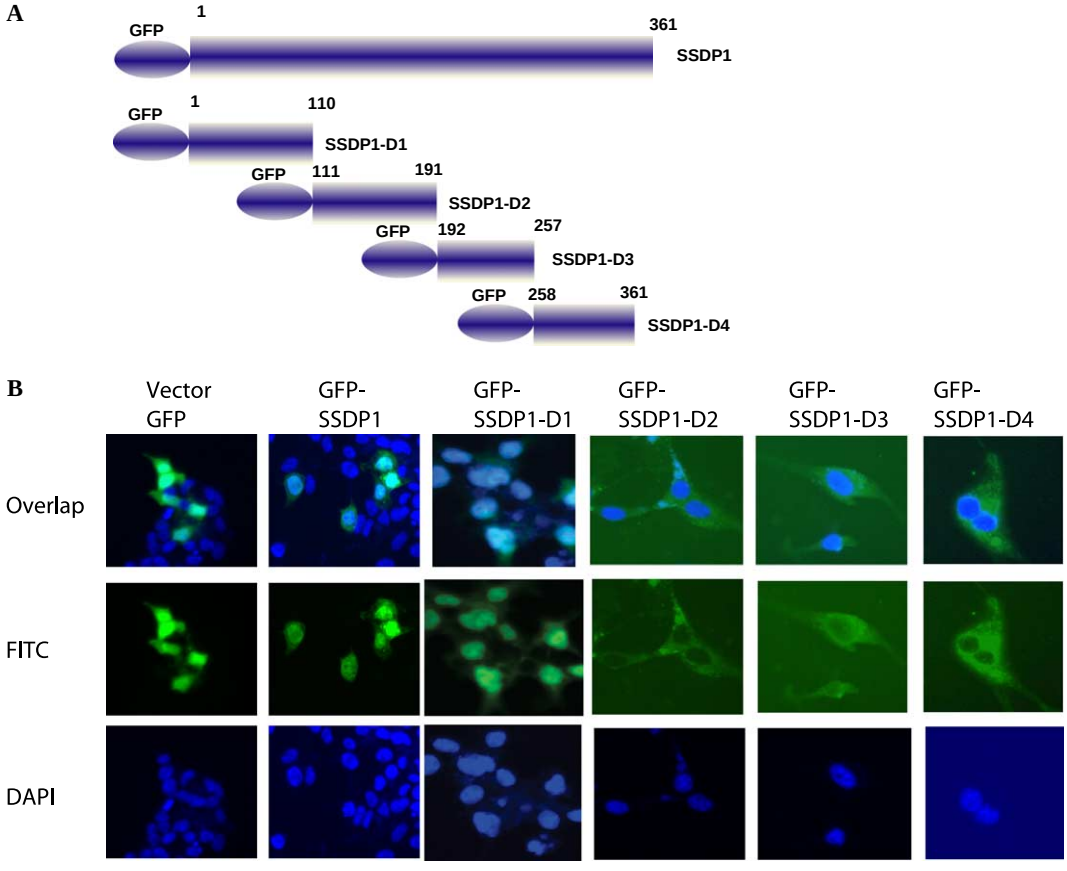

Fig. 4. Mouse SSDP1, a nuclear protein, is exclusively expressed in nuclear of subcellular localization. (A) Schematics of mouse SSDP1 and its truncated versions fused with GFP in their N-terminal. The wildly used GFP-tagged vector EGFP (BD Biosciences, Clontech) was conducted for insertion of SSDP1 and its truncated versions in-frame. (B) Mouse SSDP1 is a nuclear protein and expressed evenly. 293-T cells were transfected with either GFP vector, or GFP-SSDP1, or GFP-SSDP1-D1, or GFP-SSDP1-D2, or GFP-SSDP1-D3 or GFP-SSDP1-D4, respectively. The cells were fixed before images were taken under a microscope. The software Axiovision 4.1 allowed two channel (green and blue colors) images and overlap images taken once and exported them as tiff files. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

precise elucidations and further guideline for protein design.

SSDP family proteins are highly conserved in various species, from *C. elegans* to humans. Multiple alignments of protein sequences suggest that there are four domains in SSDP1 with high significance, represented as D1 to D4, assessed in Prodom web-based software, which provides useful tools in experimental design and implementation. These virtual data were further confirmed by my biochemical and functional studies. Therefore, bioinformatics indeed promote scientific progress.

N-Terminal of SSDP1 consists of 92 amino acids, equivalent to the LUFS domain described, which contains LisH domain, Lissencephaly type-1-like homology motif, alpha-helical motif, present in Lis1, treacle, Nopp140, some katanin p60 subunits, muskelin, tonneau, LEUNIG, and numerous WD40 repeat-containing proteins. It is suggested that LisH motifs contribute to the regulation of microtubule dynamics, either by mediating dimerization, or else by binding cytoplasmic dynein heavy chain or microtubules directly. LUFS domain of SSDP1 has demonstrated and is required for mediating the interaction with Ldb1, a transcriptional regulatory protein [6].

My data have demonstrated that C-terminal of SSDP1 has significant transcription activity. Thus, C-terminal of SSDP1 transcription activity might involve collagen protein transcriptional control, extracellular matrix function although four types of collegan proteins have many members. More interestingly, SSDP-1 transcription activity is not dependent on the Ldb1, supported by my observations that SSDP-1 can function alone in yeast cells as well truncated version of SSDP1 has as full function as full-length SSDP1 wild type in mammalian cell and yeast cells. Thus, Ldb1 interaction with SSDP1 may function as different as Lhx-Ldb1 interacting although a previous study shows that Lhx3 and Ldb1 and SSDP2 can form a ternary complex [6]. Similarly, Ldb1-SSDP1 may form independent protein complexes. To answer this question, it may need to capture multiple protein complexes by using a chemical cross-linking experiment in order to detect the components of macrocomplexes.

Nuclear protein SSDP1 binds DNA promoter for transcription downstream gene alpha (II) collagen [9]. Immunoblotting shows that abundant protein Ldb-1 is easily detected in 293-T cells (my unpublished data). Although SSDP1 does not contain any standard nuclear localization sequence (NLS), NLS signals for protein localization are not absolutely required because both protein binding partners and thier functioning areas also have significant contributions as determined by binding affinity, to its cellular localization.

Although the significance of SSDP1 in mouse embryo development has been characterized [7], my extended biochemical characterization of SSDP1 provides the guideline for further functional studies. There are so many aspects of the SSDP family that are needed for further investigations, such as SSDP binding partners (besides Ldb1) and thier roles in development. For instance, based on my data, a yeast two-hybrid system can be performed by truncating its C-terminal sequence, avoiding self-activation in the yeast cells. Since the SSDP family is highly conserved through evolution and conventional gene targeting of SSDP2 in mice is lethal, conditional knockout SSDP may be an elegant approach for further functional studies. In summary, SSDP1 is not only a binding partner for Ldb1 forming protein complex, but also a transcription factor, assessing targeted genomic DNA and transcribing downstream gene for transcription activation.

Experimental procedures

Reagents and antibodies. 3-Amino-1,2,4-triazole (3-AT) was purchased from Sigma and pTK-Rluc vector was purchased from Promega. The pCMV-Gal-4-VP14 was kindly provided by Dr. Kuan-Teh Jeang (NIAID, NIH). Yeast two-hybrid system kit was purchased from BD Bioscience (BD Clontech, CA).

Cell culture and transfection. 293-T cells were purchased from American Type Culture Collection and cultured in DMEM with 10% FBS and antibiotics. Cell transfection reagent (Fugene 6) was bought from Roche Diagnostics (Indianapolis, IN). Freshly thawed 293-T cells were cultured and split for next day transfection. Transfection method was implemented as instructed by the manufacturer's protocol. The transfected cells were lysed and subjected to experiments after 48 h transfection.

Dual-luciferase assay. Dual-luciferase kit was purchased from Promega. Briefly, after 48 h transfection, the transfected cells were washed once with PBS and then subjected to 1× passive lysis buffer. The total cell lysates were cleared by high-speed centrifugation and 20 μl cell lysates were loaded into 96-well sample plates (Polystyrene, Cat. No. 1450-581) and mixed with 100 μl firefly luciferase substrate. The luciferase activity was measured in a 1420 multilabel counter (Victor3, Wallac, Perkin-Elmer). Then, 100 μl Stop&Go buffer was added into the wells for measuring the *Renilla* luciferase activity. The data were stored and exported as *.xle file and further processed in Microsoft Excel for graphic presentation.

Yeast transformation and β-gal assay. Yeast two-hybrid system 3 kits and mouse brain cDNA library were purchased from BD Bioscience (Clontech). Full-length cDNA SSDP1 was cloned in pGBK-T7 vector and the library in pACT2 vector was amplified [10]. The pGBK-T7-SSDP1 was transformed in AH109 yeast strain according to instructions of the Matchmaker system manual and selected in -Trp plates. Sequential transformation was performed and yeast clones were screened in four dropout plates (-Trp, -Leu, -His, -Ade) in 3-AT at 15 mM concentration. The invert plates were incubated at 30 °C.

The transformed yeast clones harboring either wild type or its truncated versions were picked up and subjected to inoculating the membrane for overnight. The yeast clones were quenched in liquid nitrogen and exposed to X-gal substrate. The yeast clones turning blue within 30 min were counted as positive clones.

Subcellular localization. Mouse full-length SSDP1 and the truncated versions were inserted into pEGFP vector (BD Bioscience, Clontech). 293-T cells were transfected with GFP-SSDP1 and its mutants for 24 h and mounted on a glass slide for an additional 24 h. The cells were fixed with 4% paraformaldehyde and images were taken under a fluorescent microscope (Olympus BX60, Japan).

Acknowledgments

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