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Single-Stranded DNA-Binding Proteins Regulate the Abundance and Function of the LIM Homeodomain Transcription Factor LHX2 in Pituitary Cells

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Abstract

A family of single-stranded DNA-binding proteins (or SSBPs) has been shown to augment the function of LIM homeodomain (LIM-HD) transcription factors in embryogenesis by interaction with LIM domain-binding protein 1 (LDB1). No DNA-binding complex has been described, however, containing a LIM-HD protein, LDB1, and SSBP, and the mechanism by which SSBPs affect LIM-HD function had not been elucidated. Through use of electrophoretic mobility shift, antibody supershift, and ChIP analyses, we show that an Lhx2-Ldb1-Ssbp3 complex binds a specific element in the Lhx2 target gene *Cga* (encoding the alpha subunit of glycoprotein hormones) in the αT3-1 pituitary cell line. Using overexpression and knockdown approaches, we demonstrate that SSBP3 inhibits Lhx2 and Ldb1 turnover, stimulates assembly of this DNA-binding complex, promotes its recruitment to the *Cga* promoter, and enhances *Cga* transcription. These studies provide novel insights into the regulation of pituitary gene expression and LIM-HD function more generally.

Keywords

Single-stranded DNA-binding proteins; LIM-homeodomain proteins; protein turnover; gene expression; pituitary cells

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Introduction

LIM homeodomain (LIM-HD) proteins contain both the cysteine-rich protein interaction motif known as the LIM domain and a homeodomain mediating DNA binding. A diverse group of developmental programs are controlled by these transcription factors, including neuronal differentiation, limb, and eye formation in vertebrates and imaginal disc development in *Drosophila* [reviewed in [1,2]]. LIM domain-binding protein-1 (LDB1) interacts with LIM-HD and LIM-only (LMO) proteins, and the activity of the prototype LIM-HD protein Apterous is regulated by the levels of dLDB1 and dLMO, which competes with Apterous for interaction with Chip [[3,4]]. Thus, the stoichometry of LDB1, LIM-HD and LMO proteins is critical for assembly of the multiprotein complexes to which they contribute and, ultimately, their biological actions [[3, 5–8]].

The single-stranded DNA-binding proteins (SSBPs) are relatives of a chick protein isolated through its interaction with a single-stranded polypyrimidine sequence in the α2(I) collagen promoter [[9]]. These proteins were subsequently found to interact with LDB1 and regulate LIM-HD function in axis formation in *Xenopus*, wing development in *Drosophila*, and head morphogenesis in mice [[10–13]]. A LIM-HD/LDB1/SSBP DNA-binding complex has not been described, however, nor have the genetic targets of such complexes been identified.

We recently demonstrated that two mammalian SSBPs, Ssbp2 and Ssbp3, contribute to a DNA-binding complex in erythroid cells containing the transcription factors TAL1, E2A, and GATA-1, LIM-only protein LMO2, and LDB1 and established these two SSBPs inhibit proteasomal destruction of LDB1 and LMO2 [[14]]. Here we show that Ssbp3 belongs to an Lhx2- and Ldb1-containing complex in a mouse pituitary cell line, Ssbp3 and Lhx2 occupy the promoter of the *Cga* gene encoding the common subunit of four glycoprotein hormones, and these SSBPs prevent Lhx2 and Ldb1 turnover, increase assembly of the Lhx2-Ldb1-Ssbp complex, and regulate *Cga* gene transcription. These results have implications for pituitary gene expression and LIM-HD function more generally.

Materials and methods

Plasmid constructs

The short hairpin RNA (shRNA) expression vectors pSilencer-Ssbp2B, pSilencer-Ssbp2D, pSilencer-Ssbp3, and pSilencer-EGFP and expression vectors pEFIRES-Ldb1, pEFIRES-Ldb1(δ214–223), pEFIRES-SSBP2, and pFlag-CMV2-Ssbp3 have been described [[14]]. pCMV6-LHX2 was purchased from Origene and pEFIRES-SSBP3 constructed by transferring an *SSBP3* cDNA from pFlag-CMV2-Ssbp3 to pEFIRES-P [[15]]. A murine *Cga* promoter-luciferase reporter plasmid [[16]] was provided by Dr. Mark Roberson (Cornell University).

Cell culture and preparation of short-term transductants

αT3-1 cells were obtained from Dr. Stephen Hann (Vanderbilt University) and cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. For knockdown experiments, cells were transfected with shRNA expression vectors targeting Ssbp2, Ssbp3, or enhanced green fluorescent protein (EGFP) using Lipofectamine 2000 (Invitrogen). Puromycin (1μg/μl) was added to the medium 18 h after transfection and the cells cultured for another 48 h in selective medium. They were then incubated in puromycin-free medium for 24 h before use. Where indicated, cells were incubated with 1 μM MG132 or dimethylsulfoxide for 6 h before collection for Western blot analysis.

DNA-binding assays

Electrophoretic mobility shift analysis (EMSA) was carried out as detailed [14]. Ssbp2 and Ssbp3 antibodies have been described [17]. Immunoglobulin G (IgG, sc-2027) and antibodies to LHX2 (sc-19342X) and Ldb1 (sc-11198X) were purchased from Santa Cruz Biotechnology. Protein-DNA complexes were electrophoresed in 4% polyacrylamide gels in Tris-glycine buffer for 16 h at 4°C and the dried gels subjected to autoradiography. A pituitary glycoprotein hormone basal element (PGBE) probe identical to one used in studies of Lhx2 [18] and Lhx3 [19] DNA-binding activity had the sequence ATATCAGGTACTTAGCTAATTAAATGT.

Western blot analysis

Western blot analysis was performed as described [20]. A control antibody to Hdac2 (sc-7899) was purchased from Santa Cruz Biotechnology.

Quantitative RT-PCR analysis

Total cellular RNA was prepared using RNeasy (QIAGEN) and genomic DNA eliminated with DNase I treatment (Ambion). RNA (1 μg) was converted to cDNA with iScript (Bio-Rad) and analyzed by real-time PCR using iQ SYBR Green Supermix (Bio-Rad). Expression of *Ssbp2*, *Ssbp3*, *Ssbp4*, *Lhx2*, *Cga*, and *Ldb1* was normalized to *S16* ribosomal RNA. The sequences of the primers used were: GAATTCAATACCCTACTCCTCA (*Ssbp2*, forward), CTCCATTCCTCCTAATCCACCCATA (*Ssbp2*, reverse), GTGCTTGGCAACATTCCCCCC (*Ssbp3*, forward), TGGAGGCTGGTTTCCCATTCTG (*Ssbp3*, reverse), CACGATGGAGCCACACCACG (*Ssbp4*, forward), ACCGAGACCTCACCAGCAGGAC (*Ssbp4*, reverse), CTGTTCCACAGTCTGTCGGG (*Lhx2*, forward), CAGCAGGTAGTAGCGGTCAG (*Lhx2* reverse), AAGTCATTCAAGCTGTACTCGC (*Ldb1*, forward), and TCCAGTTCTGTAGCCGTTTGT (*Ldb1*, reverse).

Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was performed with an EZ ChIP kit (Upstate Biotechnology) and immunoprecipitated DNA analyzed by real-time PCR using iQ SYBR Green Supermix (Bio-Rad). Occupancy was quantified by subtracting the result with normal IgG from that with the indicated antibodies. The sequences of the primers used for the *Cga* promoter were TCCTGTTGAAATAATGTAATCCTGA (forward) and AGAGAGAGCATTTGGCCATT (reverse). The primers used for the *Cga* 3′ untranslated region (UTR) were TGTCACCACCTCCTCCCTAC (forward) and GGCTTTATTTCTGACGGAACC (reverse).

Protein turnover analysis

Cells were treated with 100 μM cycloheximide (CHX). Total cellular extracts obtained at the indicated times were subjected to Western blot analysis.

Autoradiographic analysis

Band intensities on autoradiographs were quantified with the ImageJ software program (National Institutes of Health; http://rsb.info.nih.gov).

Results and discussion

Previous studies identified a PGBE in the promoter of the *Cga* gene to which Lhx2 binds (18) and established the gene was regulated by Lhx2, Lhx3, and Ldb1 [19,21,22]. Although SSBPs have been shown to modulate the function of LIM-HD proteins in an Ldb1-dependent manner [10–13], a DNA-binding complex containing a LIM-HD protein, Ldb1, and SSBP has not been described. To investigate whether such a complex existed, we carried out EMSA with nuclear

extracts from the mouse pituitary cell line αT3-1. As described [18], incubation of αT3-1 nuclear extracts with a ^{32}P-labeled PGBE from the *Cga* promoter led to formation of a protein-DNA complex (Fig. 1, *arrowheads*) that was shifted significantly, although incompletely, by antibody to Lhx2 and shifted completely by antibody to Ldb1 (Fig. 1A, lanes 3 and 4, *open* circles). This complex was also retarded by antibody to Ssbp3 (Fig. 1A, lane 6) but not by rabbit IgG or antibody to Ssbp2 (Fig. 1A, lanes 2 and 5). None of the antibodies altered the migration of two other protein-DNA complexes (Fig. 1A, *filled* circles).

As both Ssbp2 and Ssbp3 contribute to the same Ldb1-containing complex in murine erythroleukemia (MEL) cells (14), we investigated their relative expression in αT3-1 cells. In contrast to MEL cells which express *Ssbp2* and *Ssbp3* at comparable levels [14], αT3-1 cells contain considerably more *Ssbp3* than *Ssbp2* mRNA (data not shown). To investigate whether Ssbp2 could incorporate into the complex if overexpressed, we introduced an *SSBP2* cDNA into αT3-1 cells and carried out EMSA. Importantly, the Ssbp2 antibody retarded a portion of the PGBE-binding complex in SSBP2-transduced (Fig. 1B, lane 7) but not vector-transduced cells (Fig. 1B, lane 2). Antibodies to both Ssbp2 and Ssbp3 were required to completely supershift this complex (Fig. 1B, lane 9), however, consistent with both SSBPs contributing to the complex. Finally, ChIP analysis revealed that Ssbp3, Ldb1, and Lhx2 antibodies, but not normal rabbit IgG, could precipitate chromatin fragments from the *Cga* promoter (Fig. 1C upper panel), but not its 3′ UTR (Fig. 1C, lower panel), establishing that Ssbp3 occupies this promoter with Lhx2 and Ldb1 *in vivo*. Together, these results show that Ssbp3 is an integral component of an Lhx2- and Ldb1-containing DNA-binding complex in mouse pituitary cells and that Ssbp2 can also incorporate into the complex if expressed at sufficient levels.

To evaluate the contribution of SSBPs to *Cga* gene expression, a luciferase reporter linked to nucleotides –507-46 of the *Cga* promoter was transfected into αT3-1 cells with and without SSBP expression vectors. Enforced expression of SSBP2 (and SSBP3) increased reporter activity in a concentration-dependent fashion, with SSBP3 slightly more active than SSBP2 (Fig. 1D). In contrast, expression of shRNA to *Ssbp3*, but not *Ssbp2* or *EGFP*, decreased reporter activity (Fig. 1E, left group). This lack of effect of Ssbp2 knockdown may be explained by its low expression in these cells and absence from the Lhx2 DNA-binding complex.

As previously observed [14,20,23], expression of Ldb1 alone in αT3-1 cells effected a dose-dependent reduction in reporter activity (Fig. 1F, lanes 1–4). With co-introduction of SSBP3, however, Ldb1 was converted from an inhibitor to activator of *Cga* expression (Fig. 1F, lanes 5–7 *vs.* lanes 1–4), although an excess of Ldb1 was able to overcome the SSBP3-mediated increase in *Cga* promoter activity (Fig. 1F, lanes 8–9). Finally, SSBP3 had little to no effect on luciferase activity when cotransfected with Ldb1(δ214–223), which lacks most of the Ldb1/Chip conserved domain (LCCD) mediating Ldb1's interaction with SSBPs (Fig. 1F, lane 12 *vs.* 13) [11,14]. These results place Ssbp3 in an Lhx2- and Ldb1-containing *Cga* DNA-binding complex, show it stimulates the activity of this complex in an Ldb1-dependent manner, and suggest that the levels of Ssbp3 are limiting, if not determining, for the complex's activity in αT3-1 cells.

We next investigated whether Ssbp3 regulated endogenous *Cga* gene expression in αT3-1 cells depleted of Ssbp2 or Ssbp3 protein by RNA interference. Quantitative RT-PCR analysis confirmed that *Ssbp2* and *Ssbp3* RNA were significantly, and specifically, reduced by *Ssbp2* and *Ssbp3* shRNA (Fig. 2A), whereas the abundance of *Lhx2* and *Ldb1* RNA was unaffected (data not shown). In association, Ssbp2 and Ssbp3 levels were reduced by 47% and 54% in *Ssbp2* and *Ssbp3* shRNA transductants (Fig. 2B), respectively. Finally, quantitative RT-PCR analysis showed that *Cga* expression was decreased in *Ssbp3*, but not *Ssbp2*, shRNA transductants (Fig. 2A), consistent with the low levels of Ssbp2 in these cells and its absence

from the Lhx2-containing DNA-binding complex. Given the extent of Ssbp3 knockdown achieved, this result likely underestimates its importance for *Cga* gene transcription.

Having shown that Lhx2, Ldb1, and Ssbp3 contributed to a common PGBE-binding complex, we investigated whether Ssbp3 had a role in its assembly. Compared to vector controls (Fig. 2C, lane 9), *Ssbp2* and *Ssbp3* cDNAs significantly stimulated (lanes 10 and 11) while *Ssbp3* (but not *Ssbp2*) shRNA reduced steady-state levels of the Lhx-Ldb1-Ssbp DNA-binding complex in αT3-1 cells (lane 6 *vs.* 8). The absence of an effect of Ssbp2 knockdown on DNA-binding activity (lane 6 *vs.*7) may, again, be due to the relatively low levels of this protein in αT3-1 cells.

To determine whether reduction in PGBE DNA-binding activity correlated with decreased occupancy of the *Cga* promoter, quantitative ChIP analysis was applied to shRNA-expressing αT3-1 cells. Knockdown of Ssbp3 significantly decreased its loading on the *Cga* promoter (Fig. 2D) and decreased recruitment of Ldb1 and Lhx2 (Fig. 2D), paralleling the decline levels in Lhx2-Ldb1-Ssbp3 DNA-binding activity observed with EMSA (Fig. 2C, lane 8). None of these proteins were detected in any abundance at the gene's 3′ UTR, with or without Ssbp3 knockdown (data not shown).

Although Lhx2 is not a substrate of Lmo2's E3 ubiquitin ligase, RING finger LIM domain-interacting protein (RLIM), [22,25], we investigated whether Ssbp3 similarly regulated Lhx2 protein turnover. Using Western blot analysis (Fig. 3A), Ldb1 and Lhx2 levels increased in Ssbp2- and, particularly, Ssbp3-transduced cells relative to vector-transduced cells, while neither *Ldb1* nor *Lhx2* mRNA was altered in SSBP2- or SSBP3-transduced cells (data not shown). In contrast, Ssbp3 knockdown decreased endogenous levels of both Lhx2 and Ldb1, whereas depletion of Ssbp2 had minimal effect (Fig. 3B). Furthermore, Ssbp3 knockdown markedly accelerated the turnover in both Ldb1 and Lhx2 protein levels in αT3-1 cells treated with the protein synthesis inhibitor CHX (Fig. 3C and 3D). Finally, to gain insight into the mechanism by which Ssbp3 overexpression increased Ldb1 and Lhx2 protein accumulation, SSBP3-transfected cells were incubated with and without a chemical proteasome inhibitor, MG132. MG132 increased the abundance of both Ldb1 and Lhx2 in αT3-1 cells similar to the effect of enforced SSBP3 expression (Fig. 3E). However, the combination of MG132 treatment and SSBP3 transfection did not increase Ldb1 or Lhx2 protein levels over that observed with either manipulation alone, consistent with their acting in series. While the ubiquitin ligase for LHX2 is not known, precluding the type of studies we carried out for LMO2 [14], these studies are compatible with Ssbp3 inhibiting Lhx2 ubiquitination and proteasomal destruction through interference with its ubiquitin ligase.

While LMO proteins must be recruited to DNA by transcription factors, LIM-HD proteins are able to bind DNA directly. To investigate Ssbp3's effect on Lhx2 affinity for DNA, cold competition studies were carried out with nuclear extracts from SSBP3- and vector-transduced αT3-1 cells. Although the overall abundance of the Lhx2-Ldb1-Ssbp3 binding complex was increased in SSBP3-transduced compared to control cells (Fig. 4A), the complex was similarly displaced from DNA with increasing amounts of unlabeled probe (Fig. 4A and quantified in Fig. 4B). Thus, Ssbp3 promoted the assembly of a DNA-binding complex to which it contributes but had no effect on the affinity of this complex for DNA.

Two LIM domain proteins, Lhx2 and Lhx3, have been shown to augment *Cga* transcription [22,25,26], and several SSBPs have been reported to regulate the activity of LIM-HD proteins [10,11]. Whether LIM-HD protein-containing complexes also contain SSBPs and Ldb1 and how SSBPs affect LIM-HD transcriptional function were unknown, however. We establish that Ssbp3, Lhx2, and Ldb1 contribute to the same DNA-binding complex in pituitary cells and that SSBP3, and SSBP2 when overexpressed, regulates the abundance of this complex, at

least in part through inhibition of Ldb1 and Lhx2 turnover. These studies have implications for regulation of pituitary gene expression and LIM-HD function.

We recently described a novel biochemical function for SSBPs in inhibiting Ldb1 and LMO2 interaction with their ubiquitin ligase RLIM. Although LIM-HD proteins are weakly ubiquitinated, if at all, by RLIM [22], the finding that SSBP3 (and overexpressed SSBP2) decreased Lhx2 protein turnover suggests that the SSBPs inhibit Lhx2's interaction with its ubiquitin ligase and explains the dose-sparing effects of mouse Ssdp1/Ssbp3 on axis duplication in *Xenopus* embryos injected with RNAs for *Xenopus* Ldb1 and the LIM-HD protein Lim1 [10].

Bach and colleagues recently reported [26] that Ldb1 protects Lhx3 from proteasomal degradation in αT3 cells and confirmed our findings that SSBPs prevent RLIM from interacting with Ldb1 and inhibit Ldb1 ubiquitination and turnover [14]. These current studies uniquely identify which SSBPs are expressed in αT3-1 cells, place Ssbp3 in the PGBE-binding complex, and elucidate the effects of SSBPs on the assembly of the complex and its affinity for DNA. This work also provides the first example of preferential utilization of an SSBP by a transcription factor complex and indicates that gene- and tissue-specific differences exist in the complexes assembled even though all three SSBPs interact with Ldb1.

Finally, while SSBP2 and SSBP3 possess unique functions [27], our studies underscore their similarity in inhibiting LMO and LIM-HD protein turnover. This likely reflects the high degree of conservation in the LUFS domain that mediates their interaction with Ldb1.

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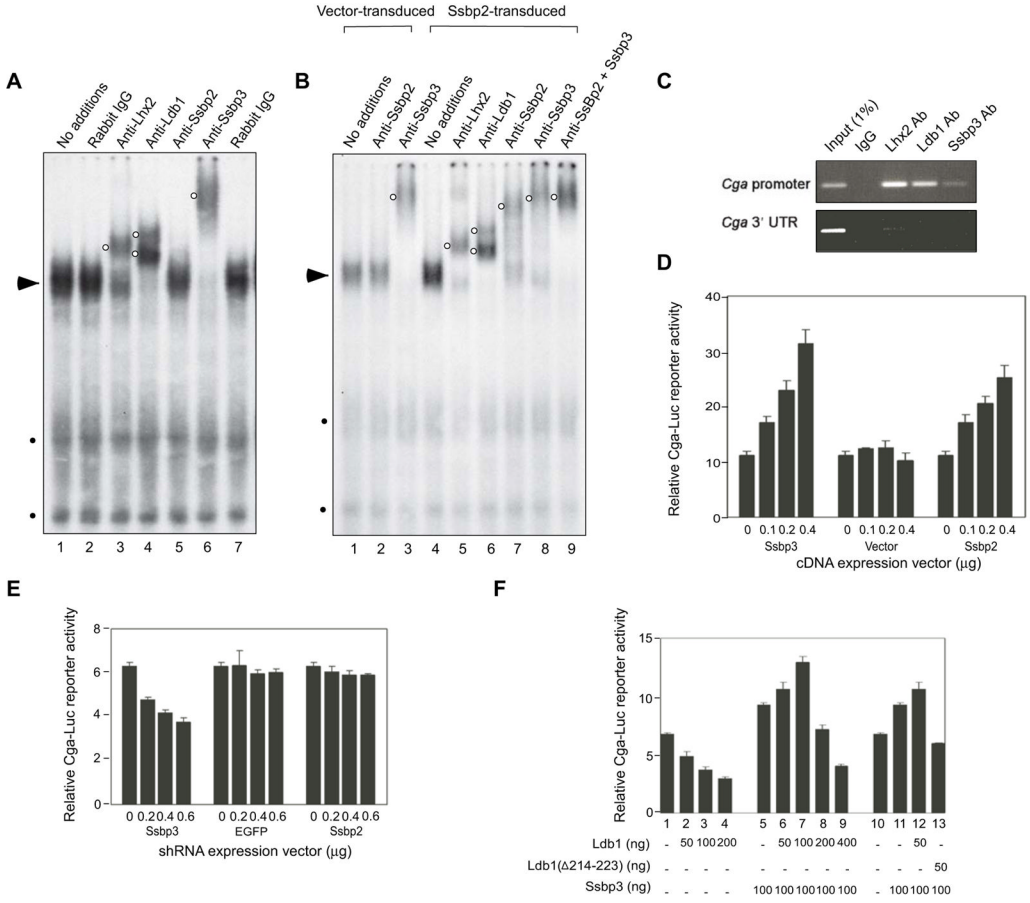

Fig. 1.

Ssbp3 is an integral component of a pituitary Lhx2- and Ldb1-containing transcriptional complex. EMSA and supershift analysis of nuclear extracts from non-transduced (A) and SSBP2-transduced αT3-1 cells (B). Ssbp3-, Ldb1-, and Lhx2-containing DNA-binding complex is marked with *arrowhead*. Complexes supershifted by Lhx2, Ldb1 and Ssbp3 antibodies are marked with *open circles.* Non-specific complexes are marked with *filled circles*. (C) ChIP analysis of Ssbp3, Ldb1, and Lhx2 occupancy of the *Cga* promoter (*upper* panel) and its 3′ UTR (*lower* panel). Transient transfection analysis with a *Cga* promoter-luciferase reporter plasmid in αT3-1 cells transduced with an *Ssbp3* or *Ssbp2* cDNA or parental vector (D) or with shRNAs to Ssbp3 (*Ssbp3* shRNA, *left*), EGFP (*EGFP* shRNA, *middle*) and Ssbp2 (*Ssbp2* shRNA, *right*) (E). (F) Reporter analysis in αT3-1 cells transduced with indicated combinations of pIRES-Ldb1, pIRES-Ldb1(Δ214-223), and pIRES-Ssbp3.

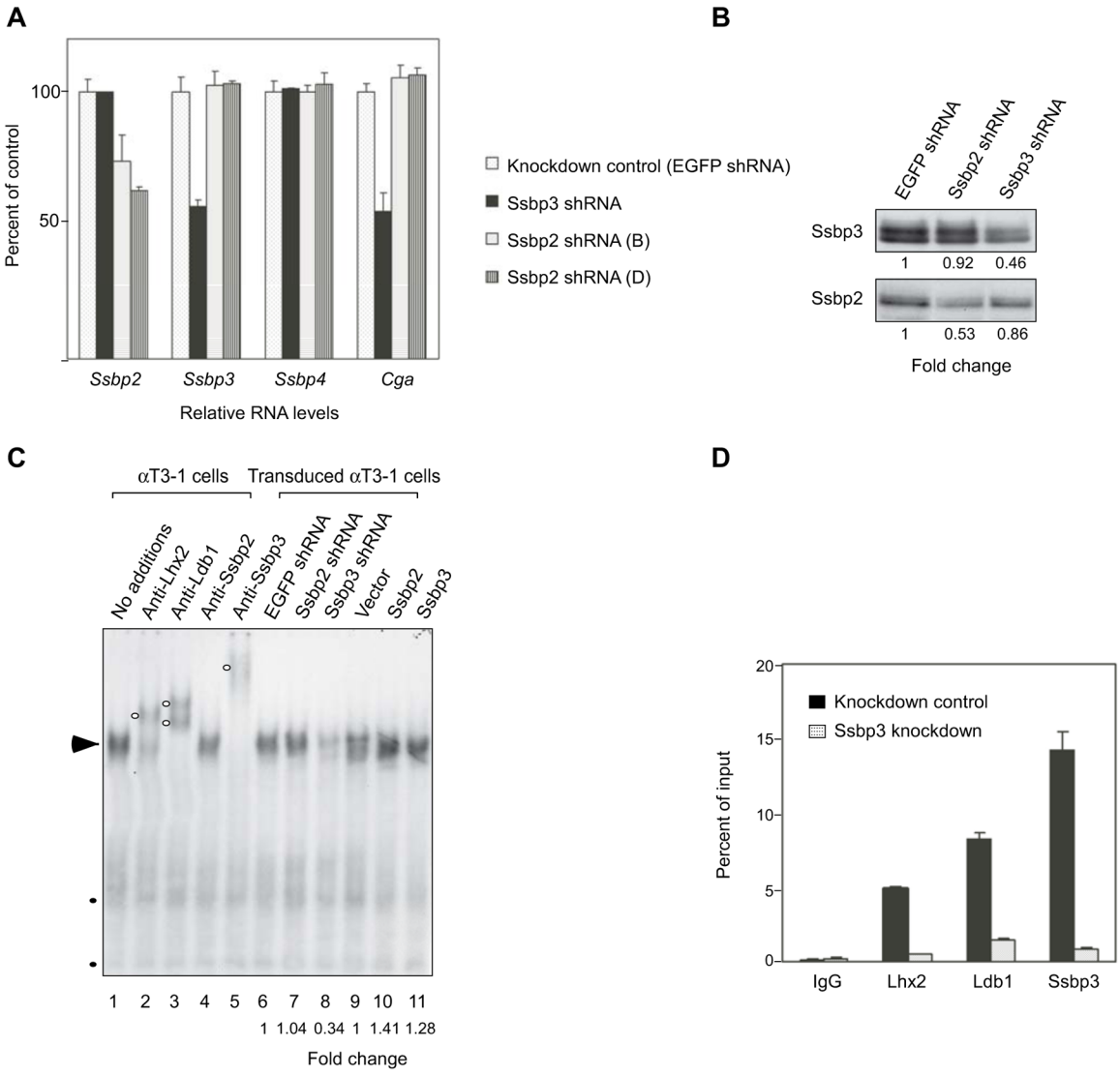

Fig. 2.

Ssbp3 regulates assembly of the Lhx2-Ldb1-Ssbp3 complex, its occupancy of the *Cga* promoter, and *Cga* gene expression in αT3-1 cells. (A) RT-PCR analysis of *S16*, *Ssbp2*, *Ssbp3*, *Ssbp4*, and *Cga* mRNAs in short-term puromycin-selected αT3-1 cells transduced with shRNAs for *Ssbp2*, *Ssbp3*, or an irrelevant RNA (EGFP). (B) Western blot analysis of Ssbp3 and Ssbp2 abundance in nuclear extracts from αT3-1 cells transduced with the indicated shRNA. (C) EMSA and supershift analysis of nuclear extracts from αT3-1 cells transduced with the indicated vectors. The Lhx2-Ldb1-Ssbp3 complex is marked with *arrowhead* and two nonspecific DNA-binding complexes used in normalization of binding activity are marked with *open circles*. The fold change in DNA-binding activity was determined from densitometry. (D) Quantitative ChIP analysis of factor occupancy on the *Cga* promoter in short-term puromycin-selected αT3-1 cells transduced with expression vectors for *Ssbp3* or *EGFP* shRNAs.

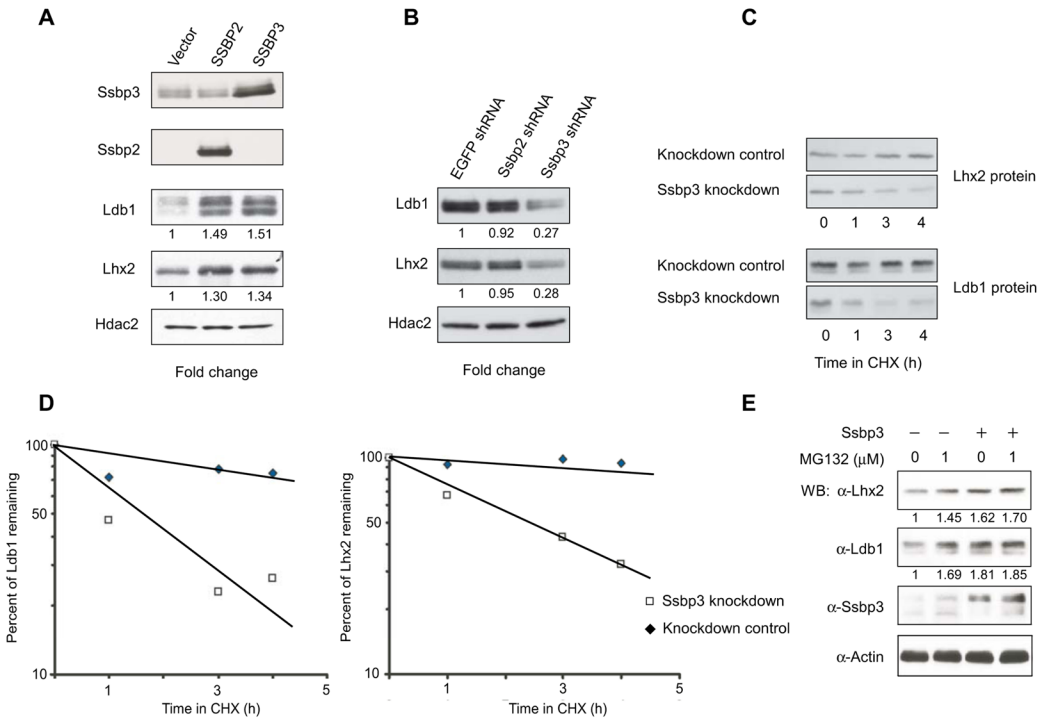

Fig. 3.

Ssbp3 regulates Ldb1 and Lhx2 protein turnover in αT3-1 cells. (A, B) Western blot analysis of Ssbp3, Ssbp2, Ldb1, Lhx2, and Hdac2 abundance in nuclear extracts from transduced αT3-1 cells. (A) Results from cells transduced with full-length *SSBP2* or *SSBP3* cDNAs. (B) Results from cells transduced with *EGFP*, *Ssbp2*, or *Ssbp3* shRNAs. (C) Protein turnover analysis in Ssbp3 knockdown and control αT3-1 cells treated with CHX (100 μM) for the indicated times. Lhx2 (*upper*) and Ldb1 (*lower*) were detected by Western blot analysis and their levels quantified by densitometry of X-ray films. (D) Percent Ldb1 and Lhx2 remaining is shown as a function of time following CHX addition. (E) Results of Western blot analysis of Ldb1, Ssbp3, and α-actin abundance in vector- and Ssbp3-transfected cells treated with 1 μm MG132 or vehicle for 6 h.

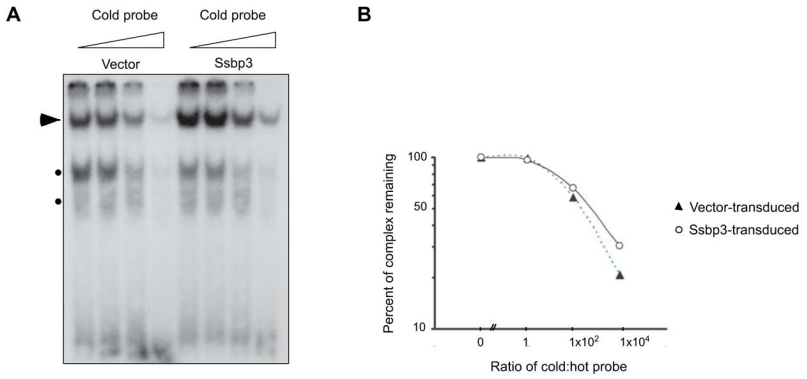

Fig. 4.

Ssbp3 does not regulate Lhx2 DNA-binding affinity. (A) The affinity of the Lhx2-Ldb1-Ssbp3 complex for DNA was assessed in pIRES-SSBP3- and vector-transduced αT3-1 cells by EMSA using increasing ratios of unlabeled to labeled PGBE probe. The Lhx2-containing complex is marked with *arrowhead*. Non-specific complexes are marked with *filled circles*. (B) Quantification of Lhx2 DNA-binding data from (A). *Open circles* denote SSBP3-overexpressing αT3-1 cells and *filled triangles* vector-transduced cells.