J. Biochem. **140**, 105–119 (2006)
doi:10.1093/jb/mvj134

Spliced Isoforms of LIM-Domain-Binding Protein (CLIM/NLI/Ldb) Lacking the LIM-Interaction Domain

Yen Ha Tran[1], Zhixiong Xu[2], Akira Kato[1,*], Abinash Chandra Mistry[1,†], Yuuki Goya[3], Masanori Taira[3], Stephen J. Brandt[2] and Shigehisa Hirose[1]

[1]Department of Biological Sciences, Tokyo Institute of Technology, Midori-ku, Yokohama 226-8501; [2]Departments of Medicine, Cell and Developmental Biology, and Cancer Biology, Vanderbilt University Medical Center and the Tennessee Valley Veterans Affairs Healthcare System, Nashville, Tennessee 37232, USA; and [3]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-0033

Received April 3, 2006; accepted May 21, 2006

LIM-domain-binding proteins (CLIM/NLI/Ldb) are nuclear cofactors for LIM homeodomain transcription factors (LIM-HDs) and LIM-only proteins (LMOs). The LIM-interaction domain (LID) of Ldb is located in the carboxy-terminal region and encoded by the last exon (exon 10) of *Ldb* genes. It is known that the mammalian *CLIM1/Ldb2* gene has a splice isoform, named CLIM1b, lacking the LID. However, little is known about the nature of CLIM1b or the evolutionary conservation of this type of alternative splicing in amphibians and teleost fish. Here, we demonstrate that splice isoforms lacking the LID are also present in the *Ldb1* genes of mammals, chick, and *Xenopus*, as well as in fish paralog *Ldb4*. All these splicing variations occur in intron 9 and exon 10. We observed that Ldb4b (splice isoform lacking LID) is localized in the nucleus when expressed in mammalian culture cells, and binds to Ldb4a (splice isoform containing LID) but not directly to LIM proteins. However, Ldb4b binds to LMO4 via Ldb4a when coexpressed in culture cells. We also found that mouse Ldb1b lacks the ability to activate protein 4.2 promoter, which is stimulated by LMO2 and Ldb1. These findings suggest that splice isoforms of Ldb lacking LID are potential regulators of Ldb function

Key words: alternative splicing, gene expression, gene structure, LIM-interaction domain, transcriptional regulation.

Abbreviations: kb, kilobase(s) or kilobase pair(s); nt, nucleotides; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; LIM domain, Lin11, Isl-1, and Mec-3 domain; LIM-HD, LIM homeodomain transcription factors; LMO, LIM-only proteins; CLIM, LIM domain–interacting coactivator; NLI, nuclear LIM interactor; Ldb, LIM domain–binding protein; LID, LIM-interaction domain; NLS, nuclear localization signal; DD, dimerization domain; Ssbp, single strand DNA binding protein; RLIM, RING-finger LIM domain–binding protein; RIPA, radioimmuno-precipitation assay; ECL, enhanced chemiluminescence.

LIM domains are double zinc-finger motifs, which have been shown to mediate protein–protein interactions that play critical roles in vertebrate development and cellular differentiation (*1–4*). In LIM homeodomain transcription factors (LIM-HDs) and LIM-only factors (LMOs), the LIM domains are required to mediate efficient interactions with other transcription factors and therefore play a key role in the regulation of transcriptional activity. The biological consequences of loss of function or misexpression of LIM-HD transcription factors are severe, demonstrating the importance of these genes in development (*5*, *6*). Members of the LIM-HD family, such as Isl1 and Lhx3, act in combination to specify neuronal cell populations. LIM-HD proteins are essential not only for the motoneuron subclass identities but also for nonneuronal cell fates (*7–10*). The requirement of LIM-HD proteins in the development of the pituitary has been demonstrated by the observation that a point mutation in the human *Lhx3* gene is associated with a combined pituitary hormone disorder (*11–13*).

LMO protein differs from LIM-HD transcription factors, which have a DNA interaction domain besides a LIM domain, in being composed almost entirely of two tandem LIM domains. Four LMOs have thus far been identified. The first two members, LMO1 and LMO2, are oncoproteins found at sites of chromosomal translocation in acute T-cell leukemia (*14*). Overexpression of two LIM-only factors (LMOs) causes acute lymphocytic leukemia (*15*). In addition, mice that possess the null mutant gene for the LIM-only protein LMO2 die on embryonic day 10.5 due to a lack of erythropoiesis, suggesting that this gene is essential for normal blood cell formation (*6*). Another member, LMO4, is highly expressed in proliferating epithelial cells and frequently overexpressed in breast carcinomas. Off-function LMO4 in mice leads to the inhibition of ductular development in virgin mice and alveolar development in pregnant mice (*16*, *17*).

There is abundant evidence that the LIM domains of the LIM homeodomain and LMO proteins interact strongly with cofactors to establish many functional regulatory complexes. The best-characterized high-affinity-binding

*To whom correspondence should be addressed. Tel: +81-45-924-5726, Fax: +81-45-924-5824, E-mail: akirkato@bio.titech.ac.jp
†Present address: Renal Division, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA.

partner for the LIM factors is the coactivator LIM-domain-binding protein Ldb/CLIM/NLI (*18–22*), which is important for either gene activation events (*20*, *23*, *24*) or inhibition of gene expression (*25*). Ldbs have been found in organisms as diverse as *Caenorhabditis elegans* and human. There are two distinct Ldb gene family members in mammals, one in *C. elegans* and *Drosophila melanogaster*, and four in zebrafish (*26*). The zebrafish *Ldb1* and *Ldb4* genes exhibit a close evolutionary relationship with the mammalian *Ldb1* gene, and the zebrafish *Ldb2* and *Ldb3* genes exhibit one with the mammalian *Ldb2* gene (*27*). The ubiquitous expression of *Ldb1* during development and its interaction with numerous transcriptional regulators indicate a critical function of the gene in a variety of developmental pathways (*18–21*, *25*, *27–30*). Elimination of *Ldb1*'s function in mice leads to severe anteroposterior patterning defects, and a lack of heart and foregut formation due to functional impediment of multiple organizer genes such as *Otx2*, *Lim1 (Lhx2)*, *Dkk1*, *Hesx1*, and *Hnf3*β during gastrulation (*31*).

Ldb has two major functional domains: a dimerization domain (DD) located in the N-terminal region and a LIM-interaction domain (LID) confined to the C-terminal region (*26*). The protein–protein interactions with Ldb are not restricted to proteins containing LIM domains, since Ldb can also bind to Ssbp (or Ssdp, single-strand DNA-binding protein), which does not have an LIM domain (*32–34*). Thus Ldb proteins act as adapter molecules facilitating the assembly of large transcriptional regulatory complexes through at least two different binding domains, and each interacting domain of Ldb has been shown to be important for the activity of the transcriptional complexes. For functional analyses of Ldb *in vivo* and *in vitro*, many deletion constructs of Ldb have been analytically generated and analyzed. One construct containing the nuclear localization signal (NLS) and LID of CLIM1/Ldb2 (amino acid residues 225 to 341) was designed and used as dominant negative CLIM (DN-CLIM). The overexpression of DN-CLIM during early developmental stages of zebrafish embryos results in impairment of eye and midbrain-hindbrain boundary development, and disturbances in the formation of the anterior midline (*35*). On the other hand, the mammalian CLIM1/Ldb2 gene has a splice isoform called CLIM1b. CLIM1b consists of DD and NLS but lacks a C-terminal LID due to a frameshift caused by the insertion of exon 9b (*20*, *36*). This domain structure suggests a potential role of CLIM1b in the regulation of CLIM1/Ldb2 function, as with other analytically generated deletion mutants. Although this type of splice isoform is very well conserved in mammalian species, little is known about the nature of CLIM1b, and the presence of similar splice isoforms lacking LID in other Ldb genes has not been reported.

Prompted by the observation that *Ldb4* in fish also has a splice isoform lacking LID similar to mammalian CLIM1/Ldb2 and in order to provide evidence for the functional activity of Ldb lacking LID, we isolated and characterized this type of splice isoform in many species ranging from fish to man. Our results demonstrate that (1) in Amniota (mammals and chicken), alternative splicing that generates CLIM1b/Ldb2b is well conserved, and *Ldb1* genes also have splice isoforms lacking LID (Ldb1b); (2) teleost fish have a well-conserved splice isoform of Ldb4 lacking LID (Ldb4b); and (3) in *Xenopus*, the *Ldb1* gene produces a splice isoform lacking LID (XLdb1b). As expected from its domain structure, Ldb4b is localized in the nucleus when expressed in mammalian culture cells, lacks the ability to bind LIM proteins, binds to Ldb4a, and indirectly binds to LMO4 via Ldb4a. We also found that Ldb1b lacked the ability to activate a promoter known to be activated by Ldb1b. Our results suggest that splice isoforms of Ldb lacking LID are potential regulators of Ldb function.

EXPERIMENTAL PROCEDURES

RNA Isolation—ICR mice and *Xenopus laevis* were obtained from local dealers. The zebrafish were a gift from Kazuyuki Hoshijima (Tokyo Institute of Technology, Japan). The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology. Total RNA was isolated from various tissues of adult animals and *Xenopus* embryos at various stages using Isogen reagent (Nippon Gene, Toyama, Japan). Chick embryo total RNA was a gift from Mikiko Tanaka (Tokyo Institute of Technology, Japan). Human fetal brain total RNA was purchased from Clontec (Palo Alto, CA, USA).

Cloning of cDNAs Encoding Novel Members of the Ldb Family—Full-length cDNAs for zebrafish Ldb4b (zLdb4b), mouse Ldb1b (mLdb1b), human Ldb1b (hLdb1b), chicken Ldb1b (cLdb1b), chicken Ldb2b (cLdb2b), *X. laevis* Ldb1b (XLdb1b), *X. laevis* Ldb2a (XLdb2a), and *X. laevis* Ldb2b (XLdb2b) were isolated by RT-PCR using KOD plus DNA polymerase (Toyobo, Osaka, Japan). The cDNA clones for zLdb4b were obtained from the gills of zebrafish using primers 5′-AGGCGCAATGCTGGATAGAGATG-3′ and 5′-ACGTAGTGGTTCTCAACTCCG-3′. The cDNA clones for mLdb1b were obtained from the hearts of mice using primers 5′-CACCATGCTGGATCGGGATGT-3′ and 5′-TGACCCCTGTCTGAGCCTGAG-3′. The cDNA clones for hLdb1b were obtained from human fetal brain total RNA using primers 5′-GATGCTGGATAGGGATGTGG-3′ and 5′-CTCACTAGCAGGCGTAGAAG-3′. The cDNA clones for cLdb1b and cLdb2b were obtained from fetal chicken total RNA using primers 5′-ATTCCACCCGGGCACCATG-3′ and 5′-AAAGGGGAGACGGTCCCGAAA-3′ for cLdb1b, and 5′-AGGCAAGATGTCCAGCGCA-3′ and 5′-ATCCACATCCTCTGAGGAGGACT-3′ for cLdb2b. The cDNA clones for XLdb1b and XLdb2b were obtained from the brains of *X. laevis* using primers 5′-AGGCACCATGCTGGATCGA-3′ and 5′-ATCCATCCTGAAAGGGAAGAGTTCC-3′ for XLdb1b, and 5′-TGGGAGCAGGCAAGAAGATG-3′ and 5′-ATCTTATTGGGAAGCTTGTTGGG-3′ for XLdb2b. The cDNA clones for XLdb2a were obtained from the hearts of *X. laevis* using primers 5′-TGGGAGCAGGCAAGAAGATG-3′ and 5′-ATCTTATTGGGAAGCTTGTTGGG-3′. The PCR products were subcloned into the *Eco*RV site of pBluescript II or pcDNA3 (Stratagene, La Jolla, CA, USA), and sequenced with an automatic DNA sequencer (LICOR 4000; LI-COR, Lincoln, NE, USA).

Plasmid Construction—cDNAs containing the entire coding regions for rat Lhx3a, mouse LMO4, and zebrafish Ldb2 were obtained by PCR using first strand cDNAs from rat pituitary, mouse brain, and zebrafish brain as templates, respectively, using KOD plus DNA polymerase (Toyobo). The sequences of the primers were

as follows: 5′-GACTACCATGCTGTTAGAAGCG-3′ and 5′-TGAGTTAGAGCCAAGGGAACTG-3′ for rat Lhx3a; 5′-GCAGACCATGGTGAATCCG-3′ and 5′-GCATTACTCTGACCTCTCAGC-3′ for mouse LMO4; and 5′-AATCAACATGTCCAACCCTCCG-3′ and 5′-GAGGCATTGTGTTTGTCTCCTAC-3′ for zebrafish Ldb2. cDNAs containing the entire coding regions for zebrafish Ldb4a, zebrafish Ldb4b, and mouse Ldb1b were obtained by PCR as described above. PCR-generated full-length cDNAs were subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA) at the *Eco*RV site and p3×FLAG-CMV-10 (N-terminal 3×FLAG-tagged protein expression vector; Sigma, St. Louis, MO, USA) at the *Eco*RV site in the right directions. The inserts of 3×FLAG-tagged expression vectors were excised again with *Eco*RI and *Bam*HI, blunt ended, and ligated with the blunt-ended *Eco*RI sites of the pCMV-Myc and pCMV-HA vectors (N-terminal myc or HA-tagged protein expression vectors, Clontech) in the right directions. All the vectors were purified using a PureYield plasmid midiprep system (Promega, Madison, WI, USA) according to the manufacturer's instructions. To generate prokaryote expression vectors, the full length cDNAs for zebrafish Ldb2, Ldb4a, and Ldb4b were subcloned into the *Sma*I site of pGEX-4T-1 (N-terminal glutathione *S*-transferase tagged protein expression vector; GE Healthcare Bioscience, Piscataway, NJ, USA) in the right directions. All the obtained constructs were verified by DNA sequencing.

Glutathione S-Transferase (GST) Pull-Down Assays—COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin under 5% CO_2/95% air at 37°C. Cells were plated at 1×10^5 cells per 35-mm dish, and 1 μg of plasmid DNA for p3×FLAG-rLhx3a or p3×FLAG-mLMO4 was transfected using Transfectin reagent (BioRad, Hercules, CA, USA) on the next day. Approximately 36 h after the transfection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in 300 μl of Triton X-100 lysis buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 1% Triton X-100, 200 μg/ml ethidium bromide, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) (*25*). After sonication and centrifugation, 80 μl of the cleared soluble extract was incubated with a 13-μl bed volume of glutathione-Sepharose 4B (GE Healthcare Bioscience) coupled to 100 pmol of GST alone or GST fusions of zLdb2, zLdb4a, or zLdb4b in 720 μl of the Triton X-100 lysis buffer at 4°C for 16 h. The protein-bound Sepharose beads were washed three times in the Triton X-100 lysis buffer and two times in the Triton X-100 lysis buffer minus ethidium bromide. The bound proteins were eluted from the beads with 80 μl of 10 mM reduced glutathione in 50 mM Tris, pH 8.0. Ten microliter aliquots of the eluates were fractionated by 10% SDS-PAGE followed by Western blot analyses using anti–FLAG M2 monoclonal antibodies (Sigma). Bound antibodies were detected using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and enhanced chemiluminescence (ECL, GE Healthcare Bioscience).

Immunoprecipitation Assay—COS-7 cells were plated at 1×10^5 cells per 35-mm dish, and 2 μg of the plasmid DNA mixture was transfected using Transfectin reagent (BioRad) on the next day. Approximately 36 h after the transfection, cells were washed twice with ice-cold PBS and then lysed in 300 μl of the Triton X-100 lysis buffer or RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS) containing 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. After sonication and centrifugation, 250 μl of the cleared soluble extract was incubated with a 13-μl bed volume of anti–FLAG M2 agarose (Sigma) at 4°C for 2 h. The protein-bound agarose beads were washed three times in the Triton X-100 lysis buffer or RIPA buffer plus 200 μg/ml ethidiumbromide, and two times in the Triton X-100 lysis buffer or RIPA buffer. The bound proteins were eluted from the beads with 50 μl of Laemmli sample buffer. Samples were reduced by the addition of 5% (v/v) 2-mercaptoethanol, and then fractionated by 10% SDS-PAGE followed by Western blot analyses using anti-HA rat monoclonal antibodies (clone 3F10; Roche Molecular Biochemicals, Mannheim, Germany), and anti-myc goat polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound antibodies were detected using peroxidase-conjugated secondary antibodies and ECL.

Fluorescence Microscopy—Transient transfection of COS-7 cells with plasmid DNA was performed with Transfectin reagent (Bio-Rad) according to the manufacturer's protocol. Cells on coverslips were fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 5% FBS in PBS for 1 h at room temperature. They were then incubated with primary antibodies (diluted to 1.2 μg/ml each) followed by appropriate secondary antibodies conjugated with Alexa 488 or 546 (1:2,000; Molecular Probes, Eugene, OR) for 30 min. Signals were captured with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The images were obtained with a high-resolution digital charge-coupled device (CCD) camera (AxioCam HRm, Carl Zeiss) and processed with AxioVision 4.1 software (Carl Zeiss).

RT-PCR—Total RNA (5 μg) was reverse-transcribed using Oligo dT primer and 200 U of SuperScript II reverse-transcriptase (Invitrogen) in a total volume of 20 μl at 42°C for 50 min. 0.125 μl of first-strand cDNA was used as a template for the PCR reaction with specific primers for either zebrafish Ldb4, *Xenopus* Ldb1, or *Xenopus* Ldb2. The sequences of the primers were as follows: 5′-CAGTTTTGCGCTCTCCAGCCAG-3′ and 5′-GGCTGGTGTACTAAAATGACGCC-3′ for zebrafish Ldb4; 5′-TGTCATCCTAGAGCCCATGCAG-3′ and 5′-GAAGCCTGTGAAGTTGGGTT-3′ for *Xenopus* Ldb1; and 5′-TACCCCGCAGCATTTTAGCCA-3′ and 5′-AAGCTTGTTGGGTCGGGTTGTC-3′ for *Xenopus* Ldb2.

Each reaction mixture consisted of 0.125 μl of cDNA, 0.2 μM each primer, 2.5 μl of 10× Ex Taq Buffer, 0.25 mM dNTP, 0.125 U of Ex Taq Hot Start version (Takara Bio, Otsu, Japan), and nuclease-free water in a final volume of 25 μl. The conditions were as follows: for zebrafish Ldb4 and *Xenopus* Ldb1, 27 or 32 cycles of denaturation (94°C, 15 s), annealing (59°C, 30 s), and extension (72°C, 1 min); for *Xenopus* Ldb2, 30 or 35 cycles of denaturation (94°C, 15 s), annealing (55°C, 30 s), and extension (72°C, 1 min). After PCR amplification, 5 μl of each reaction mixture was run on a 1.2–2% agarose gel in Tris-HCl/acetic

acid/EDTA (TAE) buffer. The gel was stained with 0.5 μg/ml of ethidium bromide, and the fluorescence image was analyzed with a Fluor Imager (FLA2000; Fuji Film, Tokyo, Japan).

Northern Blot Analysis—Full length cDNA for mouse Ldb1b was used as a probe recognizing both mRNA for Ldb1a and Ldb1b. A 666-bp cDNA fragment corresponding to intron 9 of the *Ldb1* gene was isolated by PCR and used as a probe recognizing mRNA for Ldb1b only. The sequences of the primers were as follows: 5′-TCTGGGCTGCCCCACCACTCA-3′ and 5′-AGGTCCCCAAACATAATCGG-3′ for human Ldb1b; and 5′-ACTGCTCACCCCTGACCCCTGTCTGA-3′ and 5′-AGGTCCCCAAACATAATCGG-3′ for mouse Ldb1b.

Human multiple tissue Northern blots with total RNA from different tissues were purchased from Clontech. Total RNA (20 μg) from mouse tissue or cultured cells was denatured, separated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes, and then baked for 2 h at 80°C. The membranes were prehybridized for 2 h at 65°C in PerfectHyb hybridization solution (Toyobo), and then hybridized separately with each ^{32}P-labeled probe in the same buffer at 68°C for 16 h. The blots were subsequently washed under increasingly more stringent conditions (final wash: 1× SSC and 0.1% SDS for 5 h at 60°C; 1× SSC is 150 mM NaCl and 15 mM sodium acetate, pH 7.0). Membranes were exposed to imaging plates (Fuji Film, Tokyo, Japan) in a cassette overnight. The results were analyzed using a Fuji BAS2000 Bio-image analyzer (Fuji Film).

Western Blot Analysis—Tissues were homogenized in 5 volumes of RIPA buffer containing 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A at 4°C, and centrifuged at 10,000 × *g* for 15 min at 4°C. Five microliter aliquots of the supernatants were separated by SDS-PAGE on 10% polyacrylamide gel and subjected to Western blotting using an antibody for Ldb1 (goat polyclonal, Santa Cruz Biotechnology). Bound antibodies were detected using peroxidase-conjugated secondary antibodies and ECL.

Luciferase Analysis—COS-7L cells (Invitrogen) were transfected with Lipofectamine 2000 (Invitrogen) as described previously (*24*). In brief, 1.5 μg of the pGL2-P4.2p1700-Luc reporter was cotransfected with the indicated combinations of pcDNA3.1-TAL1 (50 ng), pcDNA3.1-E47 (1.5 ng), pcDNA3.1-GATA-1 (12.5 ng), pEFIRES-LMO2 (40 ng), pEFIRES-Ldb1 and pEFIRES-Ldb1b (40 ng), and *Renilla* luciferase vector (5 ng) into COS-7L cells grown in 12-well plates (Becton Dickinson, Bedford, MA, USA). All extracts were prepared 48 h after transfection, and luciferase activities were measured with a Dual-Luciferase Reporter Assay System (Promega), the reporter activities being normalized as to *Renilla* luciferase activity. Each transfection was performed in triplicate and repeated three times.

RESULTS

Conservation of Alternative Splicing of Ldb2b/CLIM1b in Amniota (Human, Mouse, Chicken)—The presence of a splice isoform of CLIM1/Ldb2 lacking LID was initially demonstrated by Bach *et al.* as CLIM1b (*20*). Alternative use of exon 9b of the *Ldb2* gene controls the addition or loss of LID (Fig. 1A). Database analyses demonstrated that the sequence of exon 9b of the *Ldb2* gene is very well conserved among mammals and chicken: comparison of exon 9b of the mouse *Ldb2* gene with those of human, dog, cow, opossum, and chicken revealed 99, 99, 89, 92, and 93% identity in the nucleotide sequence, respectively. When exon 9b is inserted, 34–amino acid residues are added at the carboxy terminus instead of LID (Fig. 1, C and D). The sequence of the 34–amino acid residues of mouse CLIM1b/Ldb2b and those of human, dog, cow, opossum, and chicken exhibit 100, 100, 82, 85, and 85% identity in the amino-acid sequence, respectively. There is no sequence homologous to exon 9b of *Ldb2* in the genomes or EST databases of teleost fish (*Takifugu, Tetraodon*, medaka, and zebrafish) and *Xenopus*.

Cloning, Gene Structure, and Tissue Distribution of Zebrafish Ldb4b—During analyses of the cDNA library of eel gill, we isolated cDNA clones that, as the first clue, were highly homologous to Ldb1 and Ldb4 (GenBank accession no. AB250381). These clones had 17–amino acid residues (DLVGTKTCTVPELEDRS) at the carboxy terminus instead of LID. The nucleotide sequence of the homologous region exhibited 82 to 85% identity with those of mammalian Ldb1 (GenBank accession nos. NM_003893 and NM_010697), *Xenopus* Ldb1 (U74360), zebrafish Ldb1 (NM_131313), and zebrafish Ldb4 (NM_131316). Query of the BLAST program (NCBI) with the sequence encoding the 17–amino acid residues demonstrated the presence of EST clones that encode the 17–amino acid residues of zebrafish (GenBank accession nos. BI888940, CR931383, and CB364005) and three-spined stickleback (*Gasterosteus aculeatus*, DT991110). We could not find a sequence homologous to the 17–amino acid residues within EST and genome databases of mammals, chicken, and *Xenopus*. Comparison of the EST and zebrafish genome databases demonstrated that they are splice isoforms of Ldb4. Thus we concluded that teleost fish have a well-conserved splice isoform of Ldb4 lacking LID, and named it Ldb4b.

The entire coding region of cDNA for Ldb4b was cloned from zebrafish gill (GenBank accession no. AB250382), and the deduced amino acid sequence was compared with that of zebrafish Ldb4 (termed Ldb4a), as shown in Fig. 2, A to C. The nucleotide sequence encoding amino acid residues 1 to 300 of Ldb4b is 100% identical to that of Ldb4a, indicating that Ldb4a and Ldb4b are alternatively spliced products of a single gene. The exon-intron organization of the zebrafish *Ldb4* gene is shown in Fig. 2D. LID of Ldb4a was encoded by exon 10a. The coding region of the 17–amino acid tail of Ldb4b was found within the 3′-noncoding region of exon 10a, and termed exon 10b. Thus Ldb4a and Ldb4b are generated through alternative use of 3′ splice sites of intron 9.

The expression of zebrafish Ldb4b *in vivo* was demonstrated by the mRNA and protein levels. Semiquantitative RT-PCR analyses have demonstrated that mRNA for Ldb4b is expressed in various types of zebrafish tissues as well as Ldb4a (Fig. 2E). The presence of Ldb4b protein in vivo was confirmed by Western analyses using a nuclear extract of zebrafish brain (Fig. 2F).

The *Ldb4* gene of spotted green pufferfish (*Tetraodon nigroviridis*) was estimated from the genome database and compared with that of zebrafish by Harr plot analysis. Significant homology was observed around exon 10b as

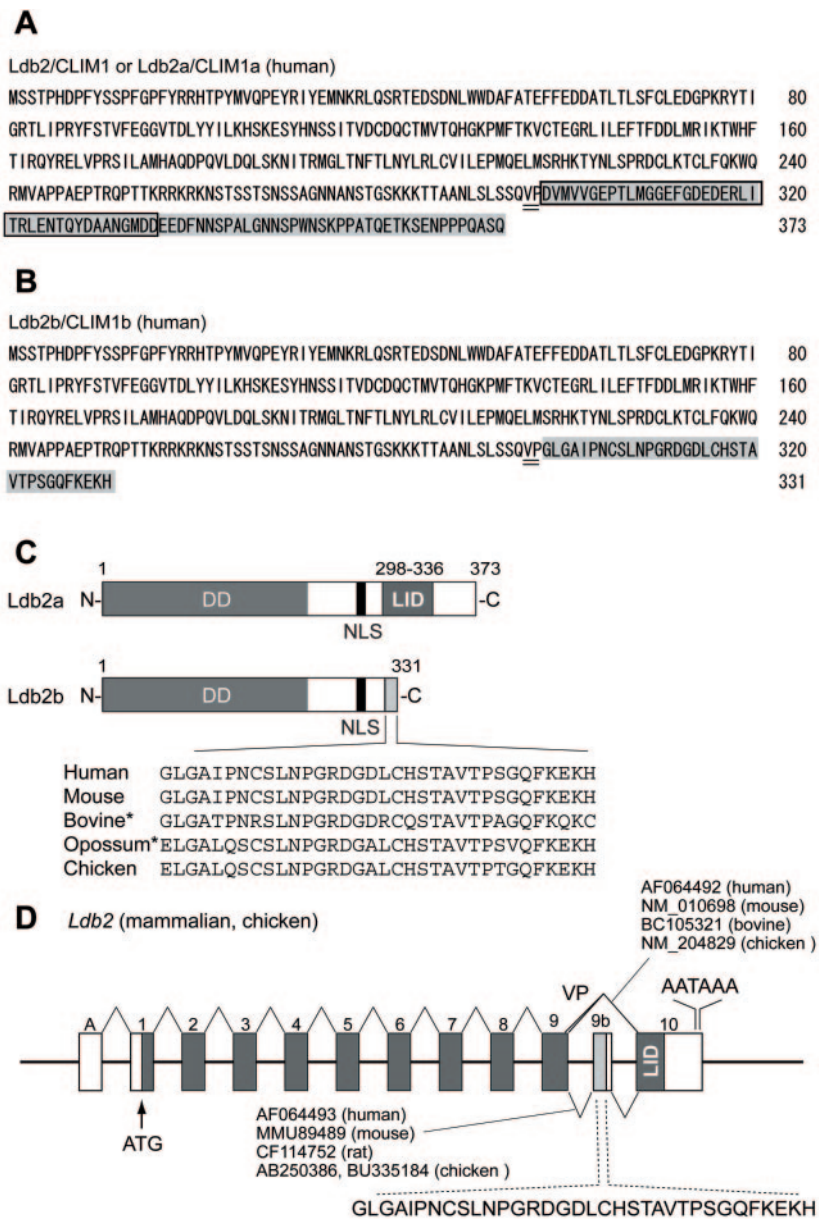

Fig. 1. **CLIM1b/Ldb2b in mammals and chicken.** The presence of a spliced isoform of Ldb2 lacking LID was demonstrated by Bach *et al.* (*20*). (A and B) Amino acid sequences of human Ldb2a (accession no. AF064492) and Ldb2b (accession no. AF064493). We confirmed that chicken also has Ldb2b (accession no. AB250386, data not shown). LID is boxed. The alternative sequences are shaded. Amino acids are numbered on the right. Another alternative splice site adding six nucleotides corresponding to the addition of two amino acids at the end of exon 9 is double underlined. (C) Schematic diagram showing the domain organization of Ldb2a and Ldb2b. The dimerization domain (DD), nuclear localization sequence (NLS), LIM-interaction domain (LID), and 34-amino-acid C-terminal sequence of Ldb2b are shown. The accession number of mouse CLIM1b/Ldb2b is NM_010698. The asterisks indicate the sequences that are only estimated from the genome databases and the presence of Ldb2b has not been experimentally determined. (D) Diagrammatic representation of alternative splicing of the human *Ldb2* gene. Ldb2a and Ldb2b are generated by skipping exon 9b. The accession numbers of the mRNA sequences of the isoforms are shown. Exons are indicated by boxes and are numbered. The introns and flanking regions are indicated by bold lines. The coding regions of alternative and common exons are indicated by dark and light gray boxes, respectively. The open boxes indicate UTRs. The arrow indicates the translational initiation site.

well as the other protein-coding exons (Fig. 3A). Exon 10b of the *Ldb4* gene was also found in the genome databases of *Takifugu rubripes* and medaka (*Oryzias latipes*), cDNA clones of rainbow trout (*Oncorhynchus mykiss*), three-spined stickleback (*Gasterosteus aculeatus*), and eel (*Anguilla japonica*) (Fig. 3B).

Identification of Mammalian and Chicken Ldb1b—The finding of Ldb4b in teleost fish raised the question of whether the mammalian *Ldb1* gene has a splice isoform lacking LID. A BLAST search of the mammalian EST and genome databases failed to reveal any sequence homologous to exon 10b of the teleost *Ldb4* gene. However, we found many EST clones in which intron 9 is retained. The accession numbers of these EST sequences are CN350347, BQ230528, and CN350347 for human; BQ963561, CA315452, and BF119088 for mouse; and DN932179, CK474935, and CK227174 for rat. All these sequences retain only intron 9, *i.e.*, they lack other intron sequences such as intron 8, eliminating the possibility of contamination by genomic or hnRNA sequences.

On RT-PCR analyses, we isolated the entire coding regions of splice isoforms of Ldb1 retaining intron 9, from human (accession no. AB250384), mouse (AB250383), and chicken (AB250385). These cDNAs only retained intron 9, *i.e.*, they lacked all the other intron sequences, and are named Ldb1b. Ldb1bs have 17 to 24-amino-acid residues at the carboxy termini instead of LID (Fig. 6, A–D).

Northern analyses of mouse Ldb1 by Agulnick and colleagues (*18*) demonstrated the presence of two major transcripts in various types of tissues. Thus we hypothesized that the upper band is Ldb1b and the lower one Ldb1a. To confirm this, we performed Northern analyses using two types of probes: one is a fragment of intron 9 that binds only to mRNA for Ldb1b, and the other is the coding region of Ldb1 that recognizes both Ldb1a and 1b. The

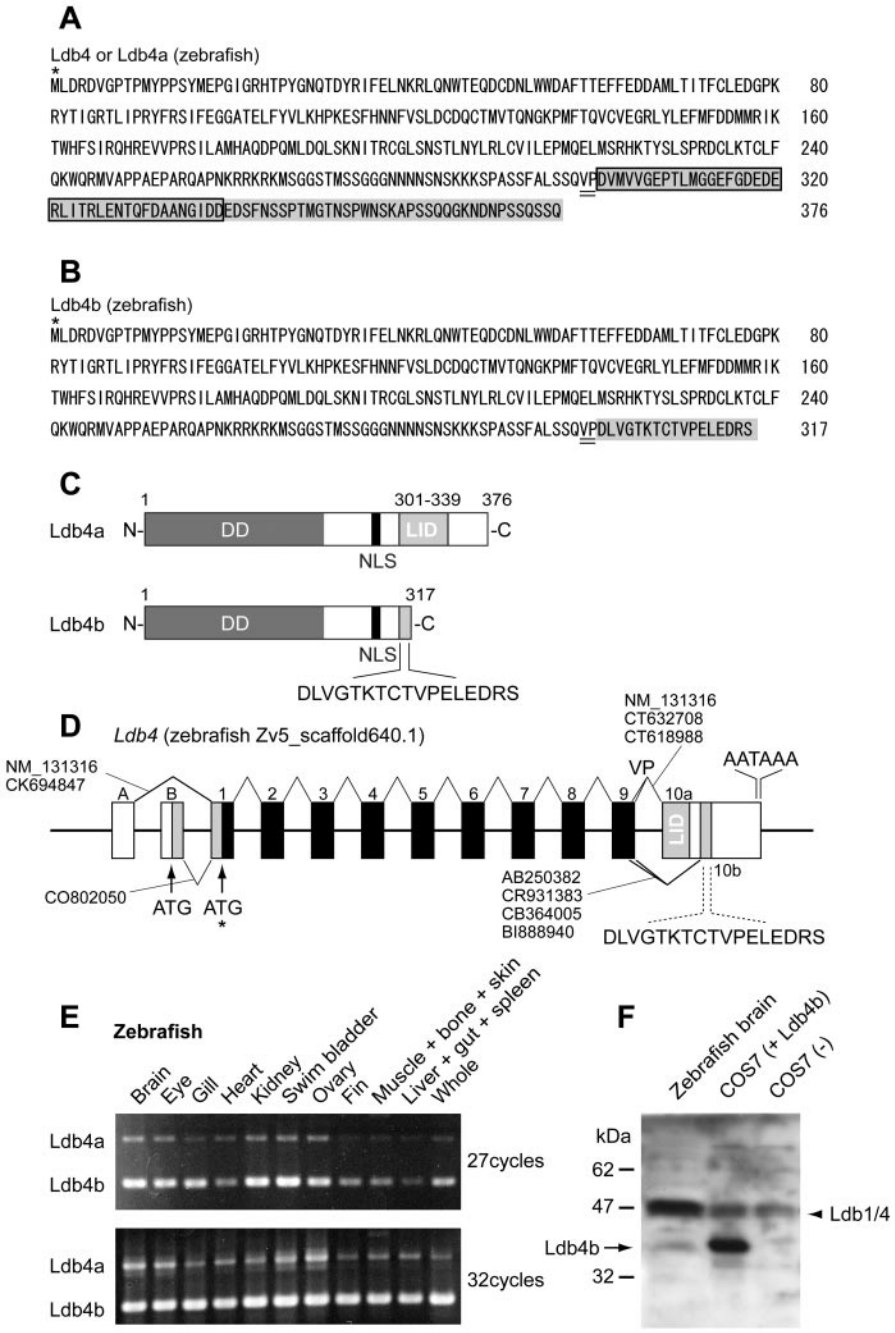

Fig. 2. **Zebrafish Ldb4b.** (A and B) Amino acid sequences of zebrafish Ldb4a (zLdb4a, accession no. NM131316) and Ldb4b (zLdb4b, accession no. AB250382). LID is boxed. The alternative sequences are shaded. Amino acids are numbered on the right. The asterisks indicate the putative translational initiation site in exon 1. Another alternative splice site adding six nucleotides corresponding to the addition of two amino acids at the end of exon 9 is double underlined. (C) Schematic diagram showing the domain organization of Ldb4a and Ldb4b. The dimerization domain (DD), nuclear localization sequence (NLS), LIM-interaction domain (LID), and 17-amino-acid C-terminal sequence of Ldb4b are shown. (D) Diagrammatic representation of alternative splicing of the zebrafish *Ldb4* gene. Ldb4a and Ldb4b are generated through alternative use of the 3′ splicing site of intron 9. The accession numbers of the mRNA sequences of the isoforms are shown. Exons are indicated by boxes and numbered. The introns and flanking regions are indicated by bold lines. The coding regions of the alternative and common exons are indicated by gray and black boxes, respectively. The open boxes indicate UTRs. The arrows indicate two different translational initiation sites, and the asterisk indicates the initiation site in exon 1 that is shown in A and B. (E) Expression of Ldb4a and Ldb4b in various tissues of adult zebrafish. Expression of Ldb4a and Ldb4b was analyzed by semi-quantitative RT-PCR. The PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide. A cDNA fragment corresponding to Ldb4b (0.86 kb) was detected for all tissues examined as well as that corresponding to Ldb4a (1.47 kb). (F) Detection of endogenous Ldb4b protein by Western blot analysis. Extracts of zebrafish brain, COS-7 cells transfected with pcDNA3-zLdb4b, and mock transfected COS-7 cells were electrophoresed, transferred to a nitrocellulose membrane, and stained with an antibody that recognizes the N-terminal of Ldb1. Bands for Ldb1/4 (arrow head) and Ldb4b (arrow) were detected at 43 and 37 kDa, respectively. Molecular size markers are shown on the left.

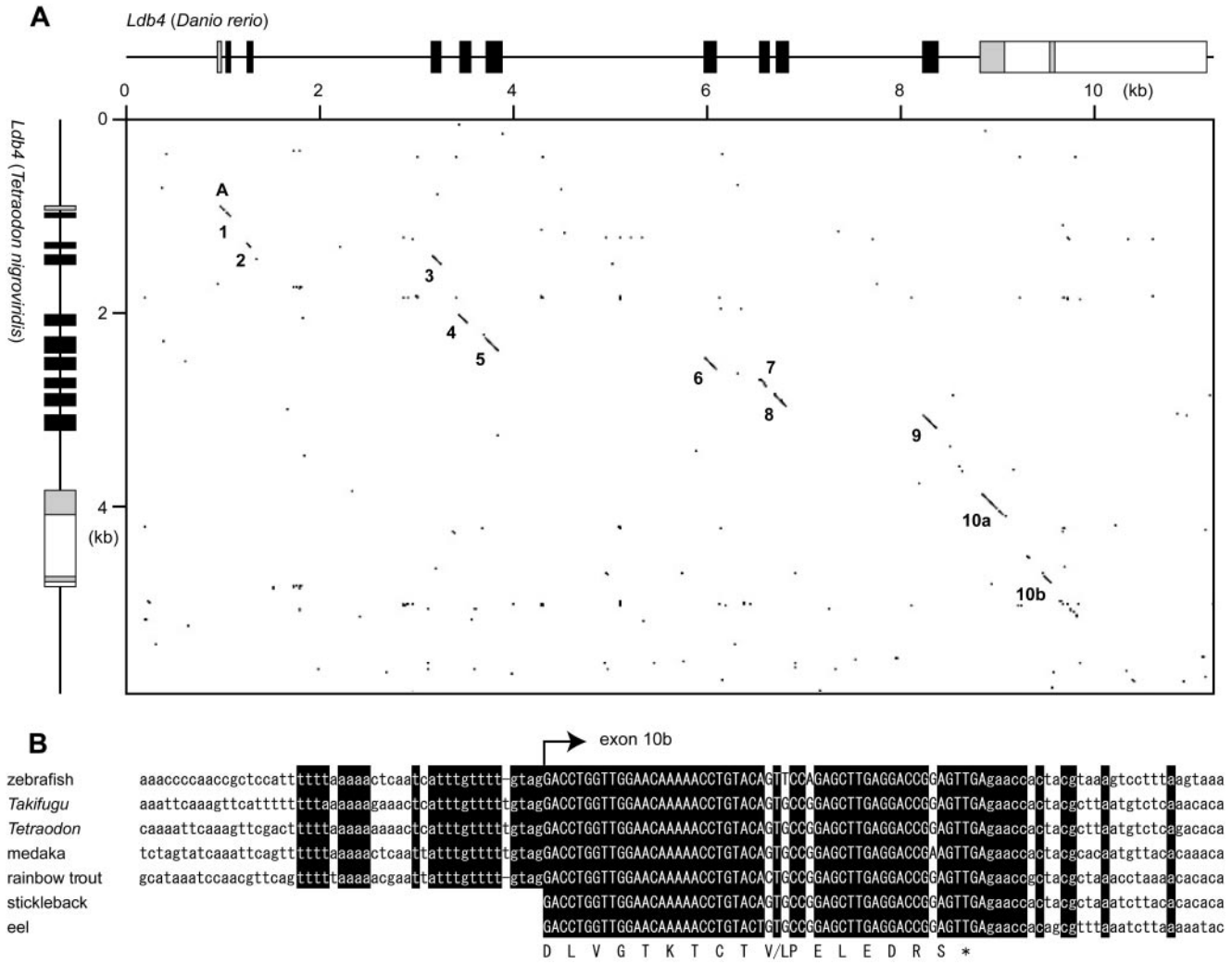

Fig. 3. **Evolutionally conserved exon 10b of teleost fish.** (A) Harr plot analyses of the zebrafish *Ldb4* gene in comparison with the *Tetraodon Ldb4* gene. The homologous regions corresponding to exons are numbered. A diagram of the exon-intron organization of each gene is shown along the axes. The coding regions of the alternative and common exons are indicated by gray and black boxes, respectively. The open boxes indicate UTRs. (B) Alignment of exon 10b. The sequences of the *Ldb4* genes from zebrafish (Zv5_scaffold640.1), *Takifugu* (scaffold M000028), *Tetraodon* (SCAF8962), and medaka (scaffold51), and the cDNA sequences of rainbow trout (accession no. BX309107), three-spined stickleback (DT991110), and eel (AB250381) are aligned. The capital letters indicate coding sequences. The arrow indicates the splice site of exon 10b.

probe for intron 9 hybridized only with the upper band (Fig. 4E), demonstrating that Ldb1b as well as Ldb1a is transcribed in various tissues. The presence of Ldb1b protein was confirmed by Western analyses using nuclear extracts of HeLa and COS-7 cells (Fig. 4F).

Cloning, Gene Structure, and Tissue Distribution of Xenopus *Ldb1b*—We also found the presence of splice isoforms of *Xenopus* Ldb1 (XLdb1) lacking LID in the GenBank database and called them XLdb1b. The accession numbers of the EST sequences are CO383533 and CO386467 for *X. laevis* Ldb1b, and DR901174, DN006626, DN006626, and CF375129 for *Xenopus tropicalis* Ldb1b. The entire coding region of XLdb1b (accession no. AB250387) was isolated and the exon-intron organization of the *XLdb1* gene was determined by comparison with a sequence in the genome database of *X. tropicalis* (Fig. 5, A–D). XLdb1a and XLdb1b are generated through alternative use of the 3′ splice site of intron 9. XLdb1b lacks LID due to the insertion of a short sequence that causes a frameshift. The amino acid sequence in place of LID is ASASP in both *X. laevis* and *X. tropicalis* (Fig. 5C).

The expression of XLdb1b *in vivo* was demonstrated by semiquantitative RT-PCR analyses. XLdb1b was expressed in various tissues of adult *X. laevis*, but the expression levels were lower than those of Ldb1a (Fig. 5E). XLdb1b was also expressed at all stages of embryo development of *X. laevis* (Fig. 5F).

Xenopus *Ldb2b Does Not Lack LID*—RT-PCR analyses of RNA from various *Xenopus* tissues demonstrated that the *Xenopus Ldb2* gene also generates two splice isoforms of its transcripts. The entire coding regions of XLdb2a (AB250389) and XLdb2b (AB250388) were isolated, and

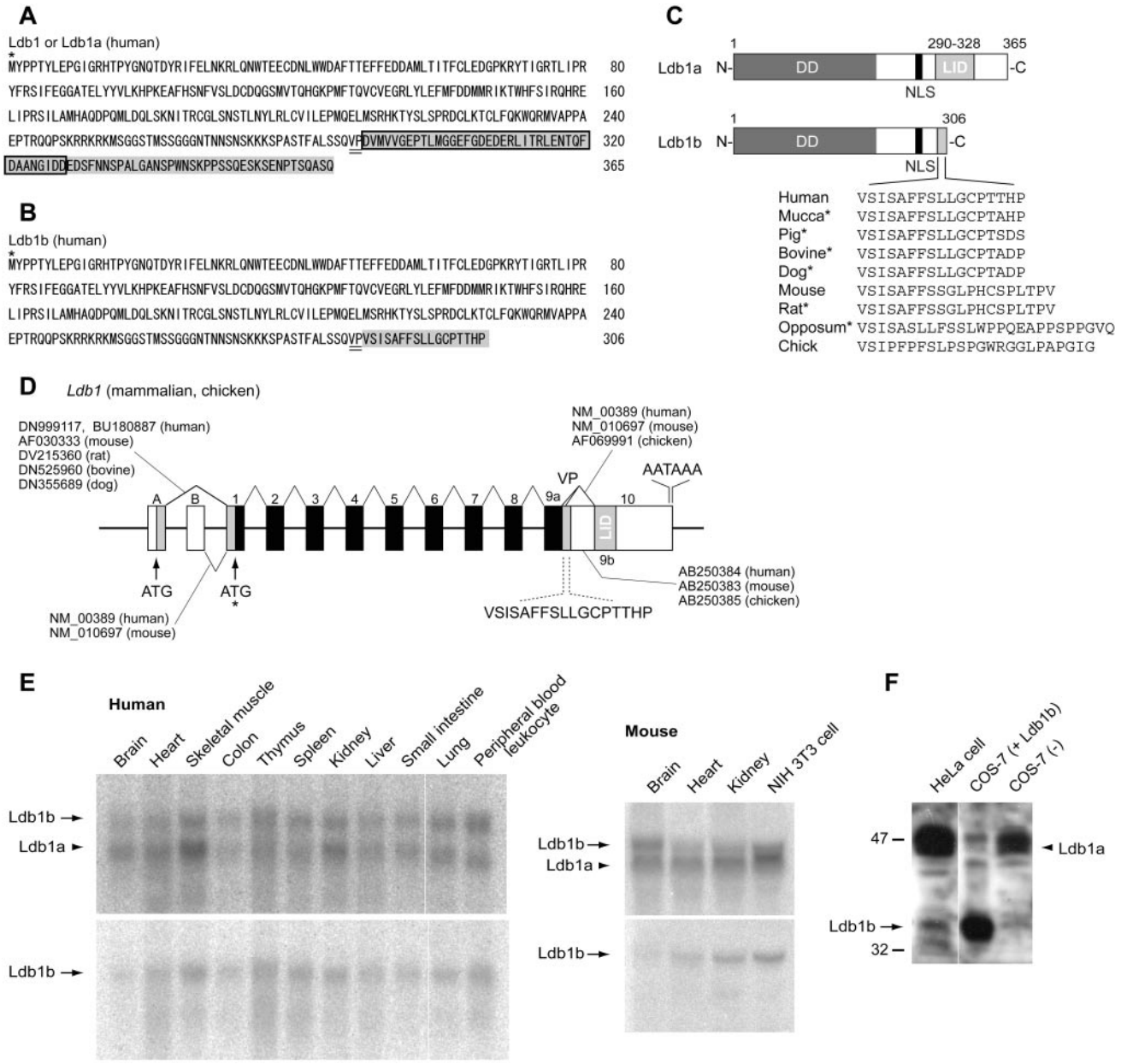

Fig. 4. **Amniota (mammals and chicken) Ldb1b.** (A and B) Amino acid sequences of human Ldb1a (accession no. NM_00389) and Ldb1b (accession no. AB250384). LID is boxed. The alternative sequences are shaded. Amino acids are numbered on the right. The asterisks indicate the putative translational initiation site in exon 1. Another alternative splice site adding six nucleotides corresponding to the addition of two amino acids at the end of exon 9 is double underlined. (C) Schematic diagram showing the domain organization of Ldb1a and Ldb1b. The dimerization domain (DD), nuclear localization sequence (NLS), LIM-interaction domain (LID), and sequence of Ldb1b at the C-terminus of various species are shown. The asterisks indicate the sequences that are only estimated from the genome databases and the presence of Ldb1b has not been experimentally determined. cDNAs for Ldb1b from human (accession no. AB250384), mouse (AB250383), and chicken (AB250385) were isolated by RT-PCR. (D) Diagrammatic representation of alternative splicing of the human *Ldb1* gene. Ldb1a and Ldb1b are generated through alternative retention of intron 9. The accession numbers of the mRNA sequences of the isoforms are shown. Exons are indicated by boxes and numbered. The introns and flanking regions are indicated by bold lines. The coding regions of alternative and common exons are indicated by gray and black boxes, respectively. The open boxes indicate UTRs. The arrows indicate two different translational initiation sites, and the asterisk indicates the initiation site in exon 1 that is shown in A and B. (E) Expression of Ldb1a and Ldb1b in various tissues of adult human and mouse. Expression of Ldb4a and Ldb4b was analyzed by Northern analyses. The nylon membranes were hybridized with a full length cDNA for Ldb1 (upper) or a 666-bp fragment corresponding to intron 9 of the *Ldb1* gene (lower). The arrows indicate bands for Ldb1b that retains intron 9, and the arrowheads indicate bands for Ldb1a that splices intron 9. (F) Detection of endogenous Ldb1b protein by Western blot analysis. Extracts of HeLa cells, COS-7 cells transfected with pcDNA3-mLdb1b, and mock transfected COS-7 cells were electrophoresed, transferred to a nitrocellulose membrane, and stained with an antibody that recognizes the N-terminal of Ldb1. Bands for Ldb1a (arrow head) Ldb1b (arrow) were detected at 43 and 35 kDa, respectively. Note that the total amount of protein loaded in the middle lane was 1/10 compared with in the other lanes, because the signal for overexpressed Ldb1b was much stronger than that of endogenous Ldb1. Thus the signal for endogenous Ldb1 in the second lane is weaker than the others. Molecular size markers are shown on the left.

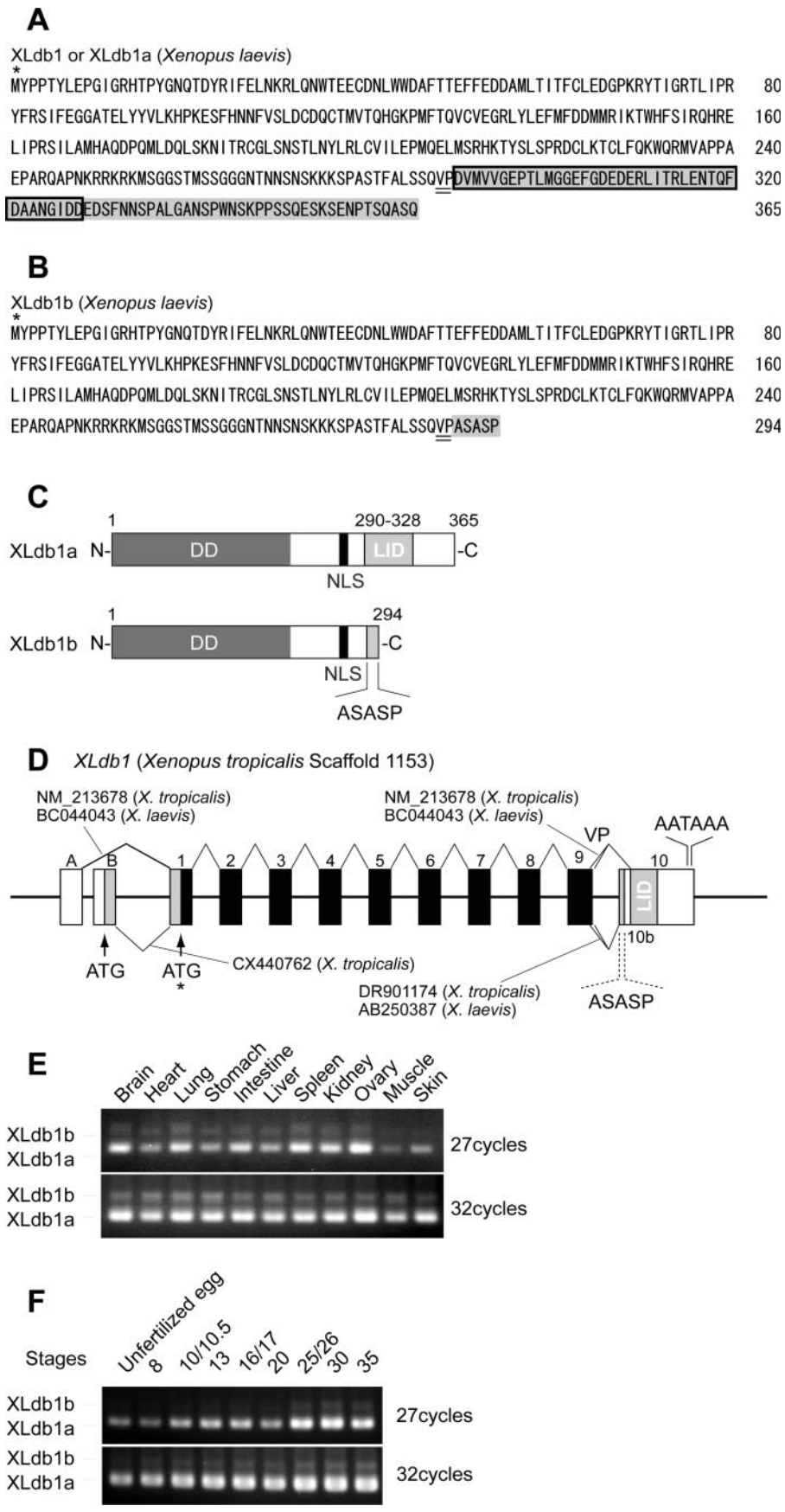

Fig. 5. ***Xenopus* Ldb1b (XLdb1b).** (A and B) Amino acid sequences of *Xenopus* Ldb1a (XLdb1a, accession no. U74360) and Ldb1b (XLdb1b, accession no. AB250387). LID is boxed. The alternative sequences are shaded. Amino acids are numbered on the right. The asterisks indicate the putative translational initiation site in exon 1. Another alternative splice site adding six nucleotides corresponding to the addition of two amino acids at the end of exon 9 is double underlined. (C) Schematic diagram showing the domain organization of XLdb1a and XLdb1b. The dimerization domain (DD), nuclear localization sequence (NLS), LIM-interaction domain (LID), and C-terminal sequence of XLdb1b are shown. (D) Diagrammatic representation of alternative splicing of the *Xenopus Ldb1* gene. XLdb1a and XLdb1b are generated through alternative use of the 3′ splicing site of intron 9. The accession numbers of the mRNA sequences of the isoforms are shown. Exons are indicated by boxes and are numbered. The introns and flanking regions are indicated by bold lines. The coding regions of alternative and common exons are indicated by gray and black boxes, respectively. The open boxes indicate UTRs. The arrows indicate two different translational initiation sites, and the asterisk indicates the initiation site in exon 1 that is shown in A and B. (E) Expression of XLdb1a and XLdb1b in various tissues of adult *X. laevis*. Expression of XLdb1a and XLdb1b was analyzed by semi-quantitative RT-PCR. The PCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide. A cDNA fragment corresponding to XLdb1b (0.60 kb) was detected for all tissues examined as well as that corresponding to XLdb1a (0.49 kb). (F) Expression of XLdb1a and XLdb1b during embryogenesis of *X. laevis*. Expression of XLdb1a and XLdb1b were analyzed by semi-quantitative RT-PCR as described above.

the exon-intron organization of the *XLdb2* gene was determined by comparison with a sequence in the genome database (Fig. 6, A–D). XLdb2a and XLdb2b are generated through insertion or skipping of exon 9b. This pattern of alternative splicing is similar to that of the *Ldb2* gene in Amniota. However, there is no significant similarity in the sequence of intron 9b between *Xenopus* and Amniota. Furthermore, the insertion of intron 9b does not cause a frameshift, and XLdb2b still has LID.

The expression of XLdb2b *in vivo* was demonstrated by semiquantitative RT-PCR analyses. XLdb2b was expressed in brain, lung, spleen, and kidney (Fig. 6E). XLdb2b was also expressed in the *Xenopus* embryo after stage 25 (Fig. 6F), and very weak expression of XLdb2b was found in the stage 10/10.5 embryo. The expression level of XLdb2b was usually higher than that of XLdb2a (Fig. 6, E–F). Heart and liver expressed low amounts of XLdb2a, but the ratios of Ldb2a:Ldb2b in these tissues were clearly different from those in other tissues (Fig. 6E).

Ldb4b Lacks LIM-Binding Ability—The binding ability of Ldb4b to LIM protein was determined by GST pull-down analyses. GST-tagged Ldb4b and Ldb4a were purified from *Escherichia coli* cells and mixed with cell extracts of COS-7 cells overexpressing FLAG-tagged Lhx3a or LMO4. The interactions were detected by Western analyses of the eluates using anti-FLAG antibodies. Lhx3a and LMO4 did not bind to Ldb4b, whereas they bound to Ldb4a and Ldb2 (Fig. 7A). This finding is consistent with the domain structure of Ldb4b, which lacks LID.

Ldb4b Is a Nuclear Protein That Binds to the Ldb4a-LMO4 Complex via *Ldb4a*—Ldb4b lacks LID but has a dimerization domain (DD) and a nuclear localization signal (NLS). This structure gave rise to the question of whether Ldb4b binds to Ldb4a in the nucleus. To confirm their interactions, myc-tagged Ldb4a and FLAG-tagged Ldb4b were coexpressed within COS-7 cells and then the interactions were analyzed by immunoprecipitation analyses. The results demonstrated that Ldb4b bound to Ldb4a (Fig. 7B). The colocalization of Ldb4a and Ldb4b in the nucleus was shown by indirect immunofluorescent analysis (Fig. 7C).

We also determined whether Ldb4b binds to the Ldb4a-LIM protein complex through Ldb4a. Myc-tagged Ldb4b was coexpressed with HA-tagged Ldb4a and FLAG-tagged LMO4 within COS-7 cells. Cell extracts were precipitated with anti-FLAG agarose and interactions were confirmed by Western analyses using anti-HA and myc antibodies. In the presence of Ldb4a, Ldb4b bound to LMO4 (Fig. 7D). However, Ldb4b did not bind to LMO4 in the absence of Ldb4a. These results indicate that Ldb4b can bind to LIM protein indirectly *via* Ldb4a.

Inhibitory Activity of Ldb1b toward Protein 4.2 (P4.2) Promoter—To understand the function of Ldb1b, we analyzed the effect of overexpression of Ldb1b on a promoter that is activated by Ldb1a. We chose the gene for protein 4.2 (P4.2), an important component of the red cell membrane skeleton, whose transcription is stimulated by LMO2 and Ldb1a (*24*). A 1.7-kb fragment of the mouse P4.2 promoter linked to a luciferase reporter gene was cotransfected into COS-7L cells with expression plasmids for Tal1, E47, GATA-1, LMO2, Ldb1a, and Ldb1b. As shown in Fig. 8, reporter activity was stimulated 24-fold by LMO2 plus GATA-1, Tal1, and E47. When Ldb1a was coexpressed with LMO2, GATA-1, Tal1, and E47, the reporter activity increased 43-fold. However, expression of Ldb1b with these proteins resulted in only a 16-fold increase in reporter activity. These results support the inhibitory activity of Ldb1b toward the P4.2 promoter. A similar inhibitory effect was observed when we used an artificially generated dimerization-defective mutant of Ldb1, which includes NLS and LID, but lacks DD (*24*).

DISCUSSION

Alternative splicing allows each gene to produce multiple protein isoforms and is one of the important mechanisms for controlling gene function (*37, 38*). Bioinformatic analyses indicated that 35 to 65% of human genes are involved in alternative splicing, which often results from differential exon inclusion, a choice between two splice sites, and intron retention (*39*). By inserting or deleting part of a protein, alternative splicing can modulate binding properties between proteins, intracellular localization, enzymatic and signaling activities, and protein stability (*38*). Many molecules involved in transcriptional regulation have splice isoforms that have different properties to promoters such as AP-2 (*40*), AML1 (*41*), Oct-2 (*42*), Lhx3 (*43*), p53 (*44*), HNF1 (*45*), ikaros (*46*), Pit-1 (*47*), and IRF-3 (*48*), suggesting that alternative splicing is among the important mechanisms regulating gene expression. In this study, we demonstrated, by inserting or deleting LID, that *Ldb* genes, whose products act as adaptor molecules of transcriptional complexes, also generate multiple proteins that show different binding properties with LIM proteins.

Table 1 is a summary of the distribution of splice isoforms of Ldb lacking LID. The splice isoforms of Ldb lacking LID are found in a variety of vertebrate animals. The splicing patterns and additional C-terminal short stretches of amino acid residues instead of LID differ among lineages such as the Amniota (mammals and chicken), amphibians (*Xenopus*), and teleost fish. In contrast, the alternative splicing of *Ldb1* and *Ldb2* in Amniota and that of *Ldb4* in teleost fish is very well conserved within each lineage in both splicing patterns and the additional C-terminal sequences instead of LID. At the coding regions for the C-terminal tail instead of LID, not only synonymous codons but also nonsynonymous codons are very well conserved (Fig. 3B). Thus we believe that the nucleotide sequences, rather than deduced amino-acid sequences, have some role in the production of two splice isoforms, and deletion of LID is more important than the amino acid sequence of the additional C-terminal short tail. It is therefore likely that the splice isoforms of Ldb lacking LID in the various animals share similar functions even though the splicing patterns and the additional C-terminal sequences are different.

It has been suggested that there are two models for the evolution of alternative splicing (*49*). The first model is the production of two or more weak splice sites, providing an opportunity for splicing machinery to produce multiple isoforms. Alternative exons possess weaker splice sites than constitutively spliced exons, and this model includes both weakening of the existing splice sites and production of a novel weak splice site. The second model represents the

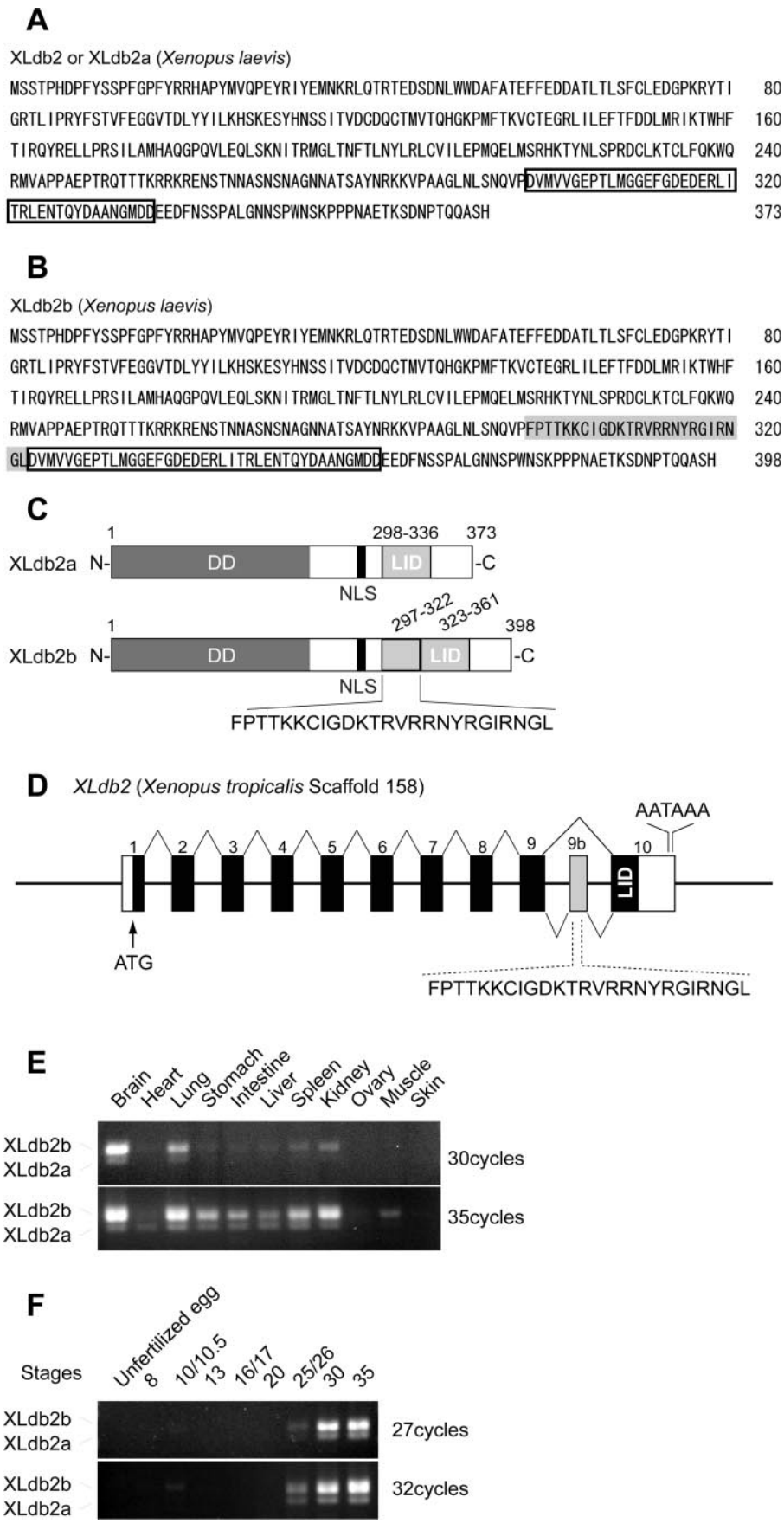

Fig. 6. ***Xenopus* Ldb2b (XLdb2b).** (A and B) Amino acid sequences of *Xenopus* Ldb2a (XLdb2a, accession no. AB250389) and Ldb2b (XLdb2b, accession no. AB250388). LID is boxed. The alternative sequences are shaded. Amino acids are numbered on the right. Another alternative splice site adding six nucleotides corresponding to the addition of two amino acids at the end of exon 9 is double underlined. (C) Schematic diagram showing the domain organization of XLdb2a and XLdb2b. The dimerization domain (DD), nuclear localization sequence (NLS), LIM-interaction domain (LID), and C-terminal sequence of XLdb2b are shown. (D) Diagrammatic representation of alternative splicing of the *X. tropicalis Ldb2* gene. XLdb2a and XLdb2b are generated through alternative use of exon 9b. Exons are indicated by boxes and numbered. The introns and flanking regions are indicated by bold lines. The coding regions of alternative and common exons are indicated by gray and black boxes, respectively. Open boxes indicate UTRs. An arrow indicates the translational initiation site. (E) Expression of XLdb2a and XLdb2b in various tissues of adult *X. laevis*. Expression of XLdb2a and XLdb2b was analyzed by semi-quantitative RT-PCR. The PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide. cDNA fragments corresponding to XLdb2b (0.64 kb) and XLdb2a (0.61 kb) are shown. (F) Expression of XLdb2a and XLdb2b during embryogenesis of *X. laevis*. Expression of XLdb1a and XLdb1b was analyzed by semi-quantitative RT-PCR as described above.

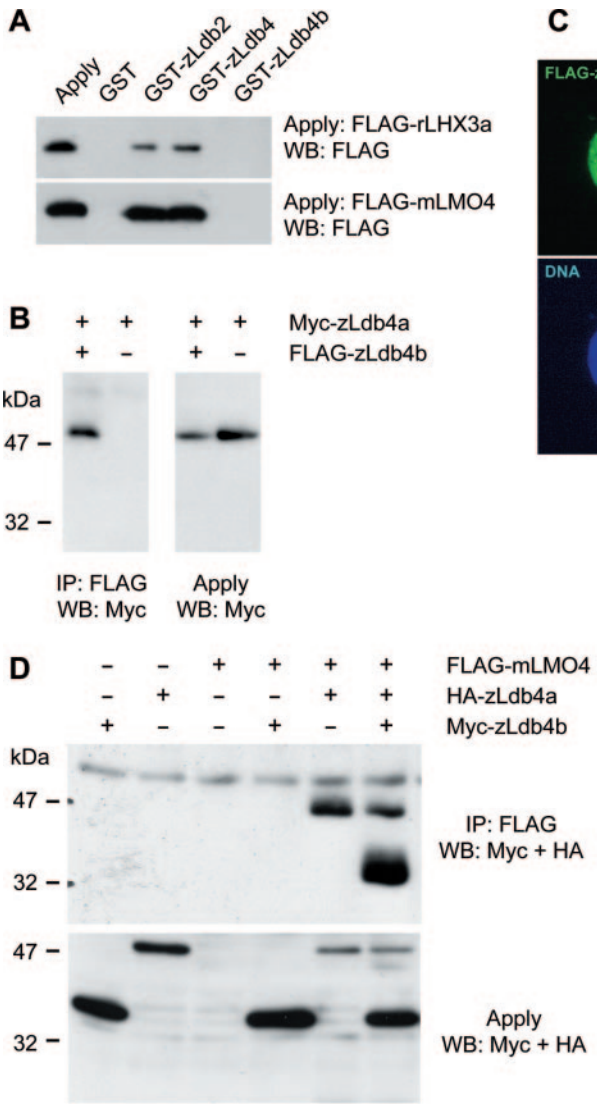

Fig. 7. **Interaction of Ldb4b with other molecules.** (A) Ldb4b lacks the ability to bind to LIM protein. GST-tagged zebrafish Ldb4b, GST-tagged zebrafish Ldb4a, GST-tagged zebrafish Ldb2, and GST only were bound to glutathione-Sepharose and mixed with an extract of COS-7 cells overexpressing FLAG-tagged rat Lhx3a or FLAG-tagged zebrafish LMO4. (B) Interaction of Ldb4b with Ldb4a. Extracts of COS-7 cells overexpressing FLAG-tagged Ldb4b alone or Myc-tagged Ldb4a were obtained. Cell extracts were immunoprecipitated with anti-FLAG agarose and the precipitates were analyzed by Western blot analyses using anti-myc antibodies. (C) Immunocytochemistry showing the co-localization of Ldb4a and Ldb4b. (D) Ldb4a-dependent interaction between Ldb4b and LIM protein. Extracts of COS-7 cells overexpresseing myc-tagged zebrafish Ldb4b, HA-tagged zebrafish Ldb4a, and FLAG-tagged mouse LMO4 were obtained. The cell extracts were immunoprecipitated with anti-FLAG agarose and the precipitates were analyzed by Western blot analyses using anti-myc and anti-HA antibodies. Ldb4b interacted with LMO4 only when co-expressed with Ldb4a.

evolution of the effect of specific splicing regulatory factors on selective use of constitutive splice sites. In our cases, the mRNAs for Ldb4b and Ldb1b are expressed ubiquitously in teleost fish and tetrapod animals, respectively, and the ratio of Ldb4a:Ldb4b or Ldb1a:Ldb1b is almost constant among the various adult tissues (Figs. 2E, 4E, and 5E). Thus far we have been unable to find any tissue or cells that show excess or reduced levels of expression of Ldb4b or Ldb1b. Therefore it is likely that both isoforms are generated through the use of two weak splice sites rather than through the effect of a specific splicing regulatory factor, and that the weakening of the splice sites occurred individually on Amniota *Ldb1*, amphibian *Ldb1*, and teleost *Ldb4* after separation of the species. In other words, it is likely that the machinery generating the splice isoforms lacking LID is common, whereas the weak splice site of each gene has developed individually. On the other hand, we found that the ratio of *Xenopus* Ldb2a:Ldb2b is different among tissues (Fig. 6E). Thus it is possible that the splicing of the *XLdb2* gene is regulated by some tissue-specific splicing regulatory factors.

It has been demonstrated that *Ldb* genes have another alternative splice site, adding six nucleotides corresponding to the addition of two amino acids (VP) at the end of exon 9 (*36*). In the current study, we demonstrated that this type of alternative splicing is also conserved among Amniota *Ldb1* and *Ldb2* (Figs. 1 and 4), amphibian *Ldb1* (Fig. 5), and teleost *Ldb4* (Fig. 2). So far there has been no report demonstrating the regulation or function of this alternative splicing.

Although the mRNA levels for Ldb4b and Ldb1b are not minor in teleost and mammalian tissues and culture cells, the protein levels of Ldb4b and Ldb1b are relatively lower than we expected (Figs. 2F and 4F). These results indicate that the translation efficiencies or stabilities of the proteins are different among the splice isoforms. As expected from the domain structure, Ldb4b lacks the ability to interact with LIM proteins such as Lhx3a and LMO4 (Fig. 7A). On the other hand, Ldb4b possesses the ability to interact with Ldb4a, possibly through the dimerization domain (Fig. 7B). Luciferase analyses have demonstrated that Ldb1b does not stimulate the P4.2 promoter, which is known to be

Table 1. **List of splice isoforms of Ldb lacking LID.**

Animals	Product name	Gene	Alternative splicing	Accession nos.
Amniota (human, mouse, chicken)	Ldb1b	*Ldb1*	retention of intron 9	human (AB250384)
				mouse (AB250383)
				chicken (AB250385)
	CLIM1b/Ldb2b	*Ldb2*	insertion of exon 9b	human (AF064493)
				mouse (MMU89489)
				chicken (AB250386)
Amphibia (*Xenopus**)	Ldb1b	*Ldb1*	alternative 3′ splice site of intron 9	*Xenopus laevis* (AB250387)
Teleostei (zebrafish, eel)	Ldb4b	*Ldb4*	alternative 3′ splice site of intron 9	zebrafish (AB250382)
				eel (AB250381)

*The *Xenopus* Ldb2 gene generates Ldb2b through the insertion of exon 9b. In *Xenopus*, the insertion of exon 9b does not cause a frameshift, and XLdb2b does not lack LID.

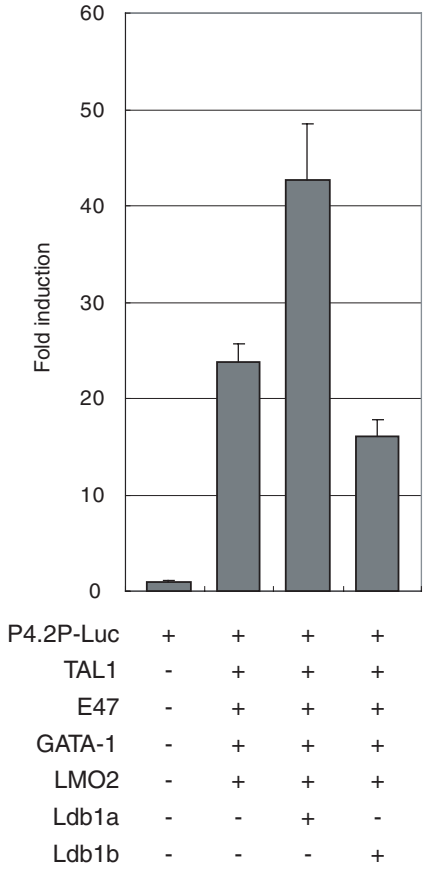

Fig. 8. **Inhibitory activity of Ldb1b toward protein 4.2 (P4.2) promoter.** A luciferase reporter construct containing the P4.2 promoter was cotransfected into COS-7L cells with different combinations of expression plasmids encoding Tal1, E47, GATA-1, LMO2, Ldb1a, and Ldb1b. Cell lysates were prepared 48 h after transfection and assayed for luciferase activity. Plotted is the mean induction in luciferase activity ± the standard error from three independent experiments.

activated by Ldb1 (*24*). Until now, only one molecule has been reported to regulate the function of Ldb1: RLIM (RING-finger LIM domain–binding protein), a RING-finger protein that ubiquitinates Ldb1 (*50–52*). We believe that Ldb1b, Ldb2b, and Ldb4b are novel regulators of Ldb's effects on LIM protein–dependent promoters. Our preliminary data suggest that Ldb1b/4b do not regulate Ldb1/4 through simple competition but may have some effects on the stability of Ldb1. The mechanisms are more complex than we expected and are likely to be elucidated in further studies.

We thank Dr. Kazuyuki Hoshijima for providing the adult zebrafish, Dr. Mikiko Tanaka for the chicken RNA, Dr. Hiroshi Sasaki for the plasmid, Dr. Gordon N. Gill for the anti-NLI antibody used in the early experiments, and Setsuko Satoh for the secretarial assistance. The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EMBL Data Bank under GenBank Accession Numbers AB250381–AB250389. This work was supported by Grants-in-Aid for Scientific Research (14104002 and 16710145) from the Ministry of Education, Culture, Sport, Science and Technology of Japan (MEXT), and the 21st Century COE Program of MEXT (to S.H. and A.K.).

REFERENCES

1. Bach, I. (2000) The LIM domain: regulation by association. *Mech. Dev.* **91**, 5–17
2. Dawid, I.B. (1998) LIM protein interactions: *Drosophila* enters the stage. *Trends Genet.* **14**, 480–482
3. Sanchez-Garcia, I. and Rabbitts, T.H. (1994) The LIM domain: a new structural motif found in zinc-finger-like proteins. *Trends Genet.* **10**, 315–320
4. Kadrmas, J.L. and Beckerle, M.C. (2004) The LIM domain: from the cytoskeleton to the nucleus. *Nat. Rev. Mol. Cell Biol.* **5**, 920–931
5. Hobert, O. and Westphal, H. (2000) Functions of LIM-homeobox genes. *Trends Genet.* **16**, 75–83
6. Warren, A.J., Colledge, W.H., Carlton, M.B., Evans, M.J., Smith, A.J., and Rabbitts, T.H. (1994) The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell* **78**, 45–57
7. Bach, I., Rhodes, S.J., Pearse, R.V., 2nd, Heinzel, T., Gloss, B., Scully, K.M., Sawchenko, P.E., and Rosenfeld, M.G. (1995) P-Lim, a LIM homeodomain factor, is expressed during pituitary organ and cell commitment and synergizes with Pit-1. *Proc. Natl. Acad. Sci. USA* **92**, 2720–2724
8. Hobert, O. and Ruvkun, G. (1998) A common theme for LIM homeobox gene function across phylogeny? *Biol. Bull.* **195**, 377–380
9. Thaler, J.P., Koo, S.J., Kania, A., Lettieri, K., Andrews, S., Cox, C., Jessell, T.M., and Pfaff, S.L. (2004) A postmitotic role for Isl-class LIM homeodomain proteins in the assignment of visceral spinal motor neuron identity. *Neuron* **41**, 337–350
10. Thaler, J.P., Lee, S.K., Jurata, L.W., Gill, G.N., and Pfaff, S.L. (2002) LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. *Cell* **110**, 237–249

11. Bhangoo, A.P., Hunter, C.S., Savage, J.J., Anhalt, H., Pavlakis, S., Walvoord, E.C., Ten, S., and Rhodes, S.J. (2006) A novel LHX3 mutation presenting as combined pituitary hormonal deficiency. *J. Clin. Endocrinol. Metab.* **91**, 743–53
12. Cushman, L.J., Showalter, A.D., and Rhodes, S.J. (2002) Genetic defects in the development and function of the anterior pituitary gland. *Ann. Med.* **34**, 179–191
13. Netchine, I., Sobrier, M.L., Krude, H., Schnabel, D., Maghnie, M., Marcos, E., Duriez, B., Cacheux, V., Moers, A., Goossens, M., Gruters, A., and Amselem, S. (2000) Mutations in *LHX3* result in a new syndrome revealed by combined pituitary hormone deficiency. *Nat. Genet.* **25**, 182–186
14. Rabbitts, T.H., Bucher, K., Chung, G., Grutz, G., Warren, A., and Yamada, Y. (1999) The effect of chromosomal translocations in acute leukemias: the LMO2 paradigm in transcription and development. *Cancer Res.* **59**, 1794s–1798s
15. Larson, R.C., Fisch, P., Larson, T.A., Lavenir, I., Langford, T., King, G., and Rabbitts, T.H. (1994) T cell tumours of disparate phenotype in mice transgenic for Rbtn-2. *Oncogene* **9**, 3675–3681
16. Visvader, J.E., Venter, D., Hahm, K., Santamaria, M., Sum, E.Y., O'Reilly, L., White, D., Williams, R., Armes, J., and Lindeman, G.J. (2001) The LIM domain gene *LMO4* inhibits differentiation of mammary epithelial cells *in vitro* and is overexpressed in breast cancer. *Proc. Natl. Acad. Sci. USA* **98**, 14452–14457
17. Wang, N., Kudryavtseva, E., Ch'en, I.L., McCormick, J., Sugihara, T.M., Ruiz, R., and Andersen, B. (2004) Expression of an engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice. *Oncogene* **23**, 1507–1513
18. Agulnick, A.D., Taira, M., Breen, J.J., Tanaka, T., Dawid, I.B., and Westphal, H. (1996) Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins. *Nature* **384**, 270–272
19. Jurata, L.W., Kenny, D.A., and Gill, G.N. (1996) Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development. *Proc. Natl. Acad. Sci. USA* **93**, 11693–11698
20. Bach, I., Carriere, C., Ostendorff, H.P., Andersen, B., and Rosenfeld, M.G. (1997) A family of LIM domain-associated cofactors confer transcriptional synergism between LIM and Otx homeodomain proteins. *Genes Dev.* **11**, 1370–1380
21. Visvader, J.E., Mao, X., Fujiwara, Y., Hahm, K., and Orkin, S.H. (1997) The LIM-domain binding protein Ldb1 and its partner LMO2 act as negative regulators of erythroid differentiation. *Proc. Natl. Acad. Sci. USA* **94**, 13707–13712
22. Valge-Archer, V., Forster, A., and Rabbitts, T.H. (1998) The LMO1 and LDB1 proteins interact in human T cell acute leukaemia with the chromosomal translocation t(11;14)(p15;q11). *Oncogene* **17**, 3199–3202
23. Mochizuki, T., Karavanov, A.A., Curtiss, P.E., Ault, K.T., Sugimoto, N., Watabe, T., Shiokawa, K., Jamrich, M., Cho, K.W., Dawid, I.B., and Taira, M. (2000) Xlim-1 and LIM domain binding protein 1 cooperate with various transcription factors in the regulation of the *goosecoid* promoter. *Dev. Biol.* **224**, 470–485
24. Xu, Z., Huang, S., Chang, L.S., Agulnick, A.D., and Brandt, S.J. (2003) Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation. *Mol. Cell. Biol.* **23**, 7585–7599
25. Jurata, L.W. and Gill, G.N. (1997) Functional analysis of the nuclear LIM domain interactor NLI. *Mol. Cell. Biol.* **17**, 5688–5698
26. Matthews, J.M. and Visvader, J.E. (2003) LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins. *EMBO Rep.* **4**, 1132–1137
27. Toyama, R., Kobayashi, M., Tomita, T., and Dawid, I.B. (1998) Expression of *LIM-domain binding protein* (*ldb*) genes during zebrafish embryogenesis. *Mech. Dev.* **71**, 197–200
28. Wadman, I.A., Osada, H., Grutz, G.G., Agulnick, A.D., Westphal, H., Forster, A., and Rabbitts, T.H. (1997) The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J.* **16**, 3145–3157
29. Dawid, I.B., Breen, J.J., and Toyama, R. (1998) LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet.* **14**, 156–162
30. Breen, J.J., Agulnick, A.D., Westphal, H., and Dawid, I.B. (1998) Interactions between LIM domains and the LIM domain-binding protein Ldb1. *J. Biol. Chem.* **273**, 4712–4717
31. Mukhopadhyay, M., Teufel, A., Yamashita, T., Agulnick, A.D., Chen, L., Downs, K.M., Schindler, A., Grinberg, A., Huang, S.P., Dorward, D., and Westphal, H. (2003) Functional ablation of the mouse *Ldb1* gene results in severe patterning defects during gastrulation. *Development* **130**, 495–505
32. Chen, L., Segal, D., Hukriede, N.A., Podtelejnikov, A.V., Bayarsaihan, D., Kennison, J.A., Ogryzko, V.V., Dawid, I.B., and Westphal, H. (2002) Ssdp proteins interact with the LIM-domain-binding protein Ldb1 to regulate development. *Proc. Natl. Acad. Sci. USA* **99**, 14320–14325
33. Nishioka, N., Nagano, S., Nakayama, R., Kiyonari, H., Ijiri, T., Taniguchi, K., Shawlot, W., Hayashizaki, Y., Westphal, H., Behringer, R.R., Matsuda, Y., Sakoda, S., Kondoh, H., and Sasaki, H. (2005) Ssdp1 regulates head morphogenesis of mouse embryos by activating the Lim1-Ldb1 complex. *Development* **132**, 2535–2546
34. van Meyel, D.J., Thomas, J.B., and Agulnick, A.D. (2003) Ssdp proteins bind to LIM-interacting co-factors and regulate the activity of LIM-homeodomain protein complexes *in vivo*. *Development* **130**, 1915–1925
35. Becker, T., Ostendorff, H.P., Bossenz, M., Schluter, A., Becker, C.G., Peirano, R.I., and Bach, I. (2002) Multiple functions of LIM domain-binding CLIM/NLI/Ldb cofactors during zebrafish development. *Mech. Dev.* **117**, 75–85
36. Ueki, N., Seki, N., Yano, K., Ohira, M., Saito, T., Masuho, Y., and Muramatsu, M. (1999) Isolation and chromosomal assignment of human genes encoding cofactor of LIM homeodomain proteins, CLIM1 and CLIM2. *J. Hum. Genet.* **44**, 112–115
37. Lopez, A.J. (1998) Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation. *Annu. Rev. Genet.* **32**, 279–305
38. Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T.A., and Soreq, H. (2005) Function of alternative splicing. *Gene* **344**, 1–20
39. Modrek, B. and Lee, C. (2002) A genomic view of alternative splicing. *Nat. Genet.* **30**, 13–19
40. Buettner, R., Kannan, P., Imhof, A., Bauer, R., Yim, S.O., Glockshuber, R., Van Dyke, M.W., and Tainsky, M.A. (1993) An alternatively spliced mRNA from the AP-2 gene encodes a negative regulator of transcriptional activation by AP-2. *Mol. Cell. Biol.* **13**, 4174–4185
41. Tanaka, T., Tanaka, K., Ogawa, S., Kurokawa, M., Mitani, K., Nishida, J., Shibata, Y., Yazaki, Y., and Hirai, H. (1995) An acute myeloid leukemia gene, *AML1*, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. *EMBO J.* **14**, 341–350
42. Lillycrop, K.A. and Latchman, D.S. (1992) Alternative splicing of the Oct-2 transcription factor RNA is differentially regulated in neuronal cells and B cells and results in protein isoforms with opposite effects on the activity of octamer/TAATGARAT-containing promoters. *J. Biol. Chem.* **267**, 24960–24965
43. Sloop, K.W., Dwyer, C.J., and Rhodes, S.J. (2001) An isoform-specific inhibitory domain regulates the LHX3 LIM homeodomain factor holoprotein and the production of a

functional alternate translation form. *J. Biol. Chem.* **276**, 36311–36319
44. Wolkowicz, R., Peled, A., Elkind, N.B., and Rotter, V. (1995) Augmented DNA-binding activity of p53 protein encoded by a carboxyl-terminal alternatively spliced mRNA is blocked by p53 protein encoded by the regularly spliced form. *Proc. Natl. Acad. Sci. USA* **92**, 6842–6846
45. Bach, I. and Yaniv, M. (1993) More potent transcriptional activators or a transdominant inhibitor of the HNF1 homeoprotein family are generated by alternative RNA processing. *EMBO J.* **12**, 4229–4242
46. Hahm, K., Ernst, P., Lo, K., Kim, G.S., Turck, C., and Smale, S.T. (1994) The lymphoid transcription factor LyF-1 is encoded by specific, alternatively spliced mRNAs derived from the Ikaros gene. *Mol. Cell. Biol.* **14**, 7111–7123
47. Sporici, R.A., Hodskins, J.S., Locasto, D.M., Meszaros, L.B., Ferry, A.L., Weidner, A.M., Rinehart, C.A., Bailey, J.C., Mains, I.M., and Diamond, S.E. (2005) Repression of the prolactin promoter: a functional consequence of the heterodimerization between Pit-1 and Pit-1β. *J. Mol. Endocrinol.* **35**, 317–331
48. Karpova, A.Y., Ronco, L.V., and Howley, P.M. (2001) Functional characterization of interferon regulatory factor 3a (IRF-3a), an alternative splice isoform of IRF-3. *Mol. Cell. Biol.* **21**, 4169–4176
49. Ast, G. (2004) How did alternative splicing evolve? *Nat. Rev. Genet.* **5**, 773–782
50. Bach, I., Rodriguez-Esteban, C., Carriere, C., Bhushan, A., Krones, A., Rose, D.W., Glass, C.K., Andersen, B., Izpisua Belmonte, J.C., and Rosenfeld, M.G. (1999) RLIM inhibits functional activity of LIM homeodomain transcription factors via recruitment of the histone deacetylase complex. *Nat. Genet.* **22**, 394–399
51. Hiratani, I., Yamamoto, N., Mochizuki, T., Ohmori, S.Y., and Taira, M. (2003) Selective degradation of excess Ldb1 by Rnf12/RLIM confers proper Ldb1 expression levels and Xlim-1/Ldb1 stoichiometry in *Xenopus* organizer functions. *Development* **130**, 4161–4175
52. Ostendorff, H.P., Peirano, R.I., Peters, M.A., Schluter, A., Bossenz, M., Scheffner, M., and Bach, I. (2002) Ubiquitination-dependent cofactor exchange on LIM homeodomain transcription factors. *Nature* **416**, 99–103