Supporting Information

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SI Methods

Cell Culture. The HeLa-based HL3T1, F1C2, and HH8 cell lines were described (1, 2, 3). The 293-based F.B3.293 and F.L7.293 cell lines were established by selecting 293 cells that expressed F.BCDIN3 and F.LARP7, respectively, by using Geneticin (Gibco).

Plasmid DNAs and siRNAs. cDNA encoding *H. sapiens* LARP7 was amplified by RT-PCR from the total RNA isolated from HeLa cells. Plasmid encoding His-tagged BCDIN3 was a gift from Dr. B. Coulombe (Institut de Recherches Cliniques de Montreal). F.LARP7 and F.BCDIN3 were expressed from the pcDNA 3.1/His^{B} plasmid (Invitrogen), in which the Xpress epitope tag was converted into the FLAG epitope tag by using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). M.LARP7, M.BCDIN3, and their derivatives contained 6 Myc epitope tags at their N-termini and were expressed from the pCS2+MT plasmid, which was a gift from Drs. S. Guo and H. Chen (University of California, San Francisco). Plasmid DNAs were transfected by using FuGENE6 reagent (Roche Applied Science). To ensure equal expressions of M.BCDIN3 and M.BCDIN3ΔBin3, the ratio of the transfected plasmid DNAs was 1:3. To ensure equal expressions of M.LARP7 and the mutant proteins with the disrupted La, RRM1, and RRM3 motifs, the ratio between the plasmid DNA encoding the wild-type protein versus the plasmid DNA encoding each mutant protein was 1:10. GST.LARP7 and its derivatives were expressed from the pGEX-6P-3 plasmid. The derivatives were constructed by site directed mutagenesis of the respective plasmid by using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Plasmid coding for 7SK snRNA was described (4). pSVED-A Tot minigene cassette was described (5). cDNA encoding *D. rerio* BCDIN3 was amplified by RT-PCR from the total RNA isolated from zebrafish embryos. cDNA encoding *D. rerio* LARP7 was obtained from Open Biosystems. To obtain RNA probes used for in situ hybridizations, both *D. rerio* cDNAs were cloned into the pCS2+MT plasmid.

siRNAs were transfected by using Lipofectamine 2000 reagent (Invitrogen). For luciferase reporter gene assay, HL3T1 cells were seeded into 6-well plates and transfected with 100 pmol of the respective siRNAs. Sixty hours posttransfection, HL3T1 cells were washed with PBS and lysed in 200 μl of reporter lysis buffer (Promega). Luciferase activity was measured in a microplate reader (LB96V MicroLumat Plus) by using Enhanced Luciferase Assay Kit (BD Biosciences). For 7SK snRNP disruptions, HeLa cells were seeded into 100-mm-diameter Petri dishes and transfected with 300 pmol of the respective siRNAs. The sequence of LARP7 siRNA was: 5′-rArArGrUrUrArArUrCrArCrCrArArArGrCrUrGrArA-3′. BCDIN3-siRNA was purchased from Sigma-Genosys and had the sequence: 5′-rGrArArCUrArCUrArCrCrGrArAUrCrCrArATT-3′. Brd4-siRNA was described previously (6). hnRNP A1 (sc-35575) and SF2/ASF (sc-38319) siRNAs were obtained from Santa Cruz Biotechnology. The control siRNA was purchased from Sigma.

Immunoprecipitation Assay and Western Blotting. Immunoprecipitations were performed as described (3). 7SK was detected by RT-PCR by using the 7SK/F and 7SK/R primers in the RNA extracted from the beads by TRIzol Reagent (Invitrogen). The sequence of 7SK/F was: 5′-CTCCAAACAAGCTCTCAAGGTCCA-3′. The sequence of 7SK/R was: 5′-ATGCAGCGCCTCATTTGGATGTGT-3′.

Immunoreagents and Chemicals. The anti-CycT1 (sc-8127), anti-Cdk9 (sc-13130, sc-484), anti-Cdk4 (sc-601), anti-c-Myc (sc-40), and anti-GST (sc-138) antibodies were obtained from Santa Cruz Biotechnology. The anti-FLAG M2 (F3165) antibody and the anti-FLAG M2 agarose beads (A2220) were purchased from Sigma-Aldrich. The anti-BCDIN3 antibody (11526–1-AP) was purchased from ProteinTech Group Inc. The anti-HEXIM1 antibody was described previously (4). The anti-LARP7 antibody was described in ref. 7. Actinomycin D (A 1410) was purchased from Sigma-Aldrich. RNase A (70194Y) was obtained from USB Corp.

Recombinant Protein Purification. GST.LARP7 proteins were expressed in the BL21(DE3)pLysS strain of *E.coli* (Novagen) after induction with 0.2 mM IPTG at room temperature and were purified by using glutathione-Sepharose beads (GE Healthcare). For EMSAs, GST was cleaved from the GST.LARP7 chimeras by using PreScission Protease (GE Healthcare).

RT-qPCR Assay. Total RNA from cells or zebrafish embryos was isolated by using TRIzol Reagent (Invitrogen). Reverse transcription was performed with the M-MLV reverse transcriptase (Invitrogen) by using random hexamers or gene-specific reverse primers. For each mRNA quantified, primers were designed by using Integrated DNA Technologies PrimerQuest program (see Table S6 for sequences). qPCR was performed with Stratagene Mx3005P real-time PCR system and SYBR Greene Jumpstart Taq ReadyMix reagent (Sigma). For experiments in HeLa cells, the values were normalized to those of GAPDH mRNA. For experiments in zebrafish, the values were normalized to those of ribosomal protein L4 mRNA.

ChIP Assay. ChIP was performed on chromatin from HeLa cells that expressed pSVED-A Tot together with control-, BCDIN3-, or SF2/ASF-siRNA. Cross-linking was achieved by incubating the PBS-washed 7×10^5 HeLa cells in 1% formaldehyde in PBS for 10 min at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were then pelleted in a conical tube and washed with cold PBS. The cell pellets were then resuspended in 1 ml of Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) for 10 min on ice and subjected to sonication to obtain DNA fragments averaging ≈200–500 bp in length. One-fifth of the total chromatin solution was used for each ChIP. Chromatin solutions were precleared with protein A Agarose/Salmon Sperm DNA beads (Upstate) and then incubated with the appropriate antibody at 4 °C overnight. Antibodies included the nonspecific rabbit polyclonal antibody, the rabbit polyclonal anti-Cdk9 (sc-484) antibody, the rabbit polyclonal anti-SF2/ASF (sc-28724), and the rabbit polyclonal anti-Ser2-P RNAPII antibody (ab5095). Protein A Agarose/Salmon Sperm DNA beads were then added, and the mixture was incubated for an additional 1 h. The beads were then washed as described in the instructions for ChIP Assay Kit (Upstate). Afterward, the immune complexes were eluted from the beads 2 times with the elution buffer (1% SDS and 0.5% NaHCO3) for 15 min at RT. The DNA–protein complexes were treated with proteinase K, followed by reverse cross-linking at 65 °C for 4 h. DNA was extracted with phenol-chloroform-isoamylalcohol, precipitated with ethanol, and dissolved in 30 μl of Tris-EDTA buffer. Each sample was diluted 3 times and 0.8 μl of DNA was used with the appropriate primer sets (for sequences, see Table S7) specific for the intergenic

genomic region, which lies 4 kb upstream of the *actin* gene (In), promoter (Pr), and exonic (Ex) regions of the minigene in qPCRs. Results were normalized to input DNA and presented as percent of input.

Liquid Chromatography with Tandem Mass Spectrometry Detection (LC MS/MS). In-gel digestion of proteins was performed by using In-gel tryptic digestion kit (Pierce). Mixtures of proteolytically generated peptides were analyzed by nanoLC MS/MS by using a 2DLC nanoHPLC System (Eksigent) interfaced with the Q-Star XL mass spectrometer (Applied Biosystems/Sciex) equipped with the nanospray II source (Applied Biosystems/Sciex). External calibration was performed in MS/MS mode by using fragment ions of Glu-fibrinopeptide as references. An LC Packings Pepmap C18 trap column (300 μm i.d., 5-mm length, 300 Å pore size, 5 μm particle size) and a column (75 μm i.d., 15-cm length) self-packed with Jupiter Proteo C12 end-capped material (90 Å pore size, 4 μm particle size) were used for desalting and reversed phase peptide separation, respectively. A 20-min linear gradient from 2% B to 50% B was run at 250 nL/min flow rate, by using solvent A (2% acetonitrile/0.1% formic acid) and solvent B (80% acetonitrile/0.08% formic acid). Precursor ion selection used an automated routine (IDA, Analyst QS 1.1. Applied Biosystems/Sciex) that consisted of a series of 1 survey MS scan (1 s, m/z 400-1700) and 2 MS/MS scans (1 s, m/z 60–1500); nitrogen served as a collision gas and collision energy was automatically adjusted depending on the size and charge state of the precursor ion. Protein identification was accomplished by using the MASCOT 2.0 (Matrix Science) search engine. Mammalia taxonomy was searched within the MSDB 20060831 (3,239,079 sequences; 1,079,594,700 residues) by using the following parameters: precursor ion mass tolerance, 150 ppm; fragment mass tolerance, 0.15 Da; tryptic digestion (including cleavages before Pro) with 3 missed cleavages; fixed modifications, S-carboxyamidomethyl; variable modifications, Carboxyammidomethyl (N-term 57), deamidation (Asn and Gln); Met-sulfoxide; Pyro-Glu (from N-terminal Gln); phosphorylation of Ser and Thr.

Fish Stocks and Maintenance. Fish breeding and maintenance were performed as described (8). Embryos were raised at 28.5 °C and staged as described (9).

In Situ Hybridization. RNA in situ hybridization was performed as described (8). Differential interference contrast microscopy was performed on a Zeiss Axiophot 2 microscope.

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9. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* 203:253–310.

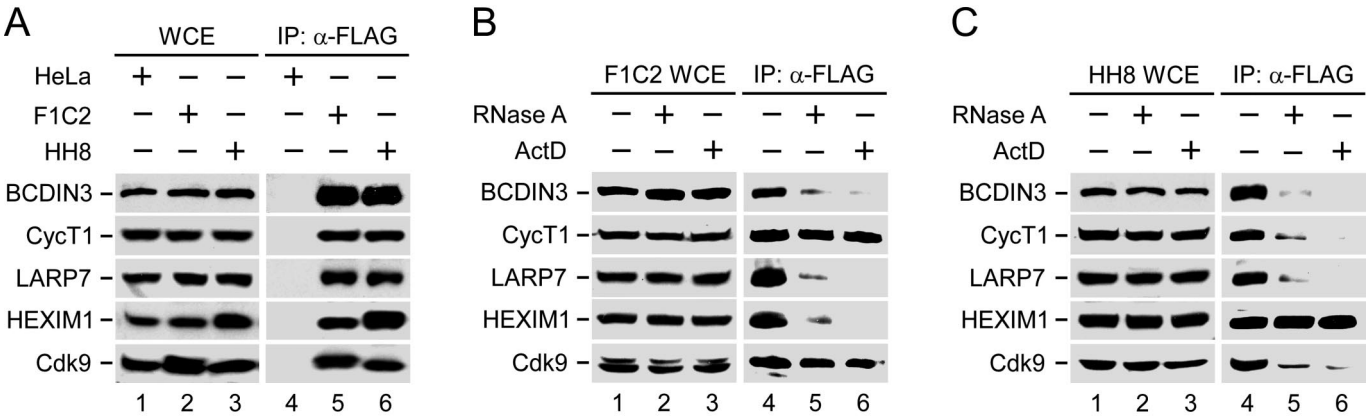

Fig. S1. LARP7 and BCDIN3 are components of 7SK snRNP, which interact with P-TEFb and HEXIM1 in RNase A- and cell stress-dependent manner. (*A*) WCEs of HeLa cells or 2 HeLa-based cell lines expressing F.Cdk9 (F1C2) and F.HEXIM1 (HH8) proteins were subjected to IP with α-FLAG as indicated. The levels of BCDIN3, CycT1, LARP7, Hexim1, and Cdk9 in WCEs (*Left*) and immunoprecipitates (*Right*) were detected by Western blotting. (*B*) Whole cell extracts (WCEs) of a HeLa-based cell line expressing F.Cdk9 (F1C2) protein were subjected to immunoprecipitations (IP) with α-FLAG M2 agarose beads (α-FLAG) as indicated. The levels of BCDIN3, CycT1, LARP7, Hexim1, and Cdk9 in WCEs (*Left*) and immunoprecipitates (*Right*) were detected by Western blotting. WCEs or cells before lysis were incubated (+) or not (−) with RNase A or ActD, respectively, as indicated above the Western blots. (*C*) WCE of a HeLa-based cell line expressing F.HEXIM1 (HH8) protein were subjected to IP with α-FLAG as indicated and analyzed as in *A*. WCEs or cells before lysis were incubated (+) or not (−) with RNase A or ActD, respectively, as indicated above the Western blots.

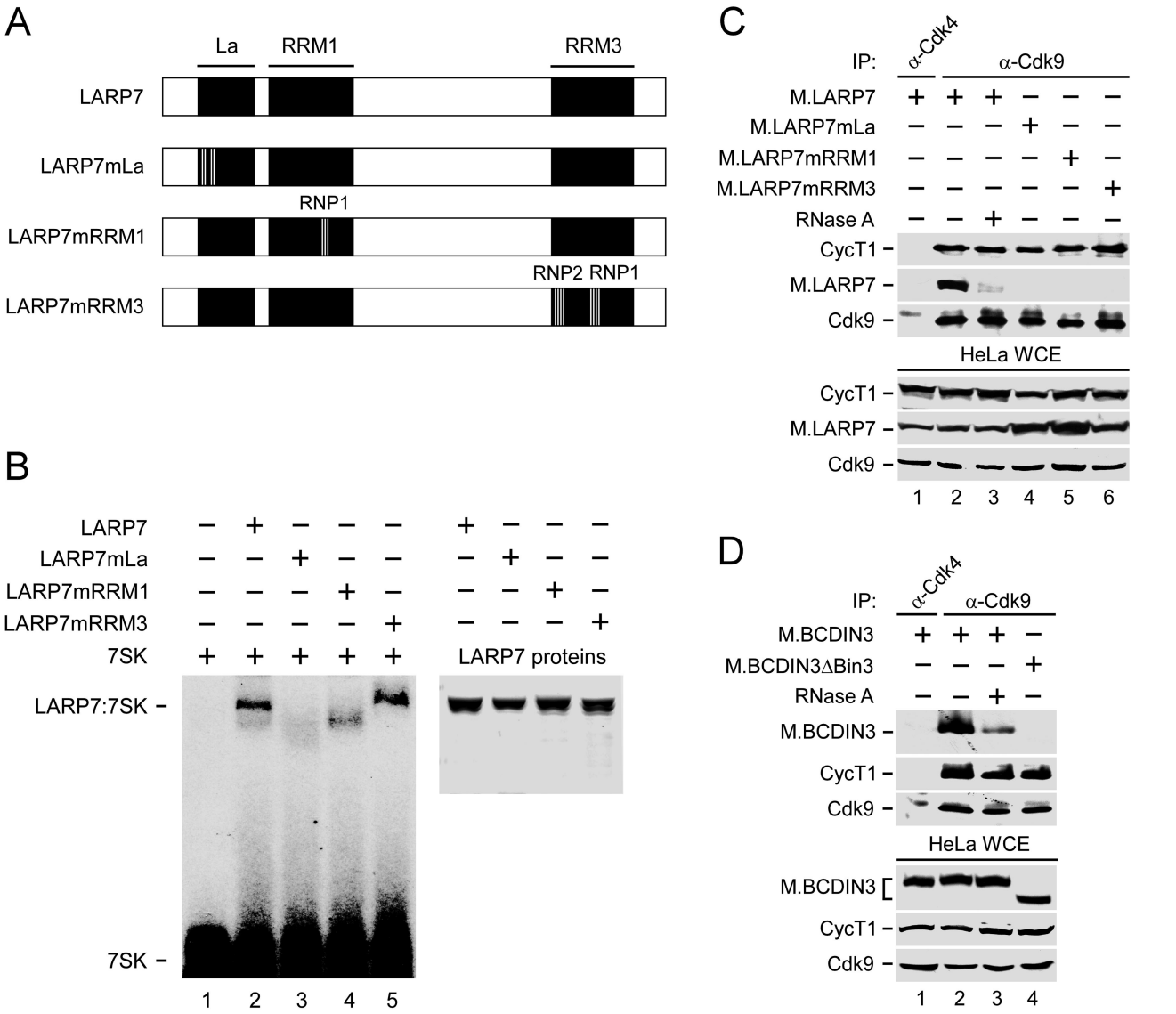

Fig. S2. Identification of motifs in LARP7, critical for 7SK binding, and interacting surfaces of LARP7 and BCDIN3, which are required for their sequestration into 7SK snRNP. (*A*) Schematic representations of the wild-type LARP7 protein with its motifs in black and the LARP7 mutant proteins containing point mutations in La, RRM1 and RRM3 motifs. LARP7mLa (Q41A, F44A, D54I, F56A) bears mutations of the conserved residues that are required in La for its interaction with the transcripts containing the UUU-OH 3′ ends [Dong G, Chakshusmathi G, Wolin SL, Reinisch KM (2004) Structure of the La motif: A winged helix domain mediates RNA binding via a conserved aromatic patch. *EMBO J* 23:1000–1007]. Similarly, LARP7mRRM1 (K166A, F168A, F170A) contains mutations within the LARP7 ribonucleoprotein 1 (RNP1) motif, and LARP7mRRM3 has mutated conserved residues within both RNP2 (V457A, K458A, I459A, I460A) and RNP1 (E492A, H494A, F497A) motifs. (*B*) Purified LARP7 proteins visualized by coomassie staining (*Right*) were incubated with α-^{32}P-labeled 7SK in EMSAs as indicated above the autoradiograph (*Left*). (*C*) WCEs of HeLa cells that expressed the indicated M.LARP7 proteins were subjected to IP with the antibodies as shown (*Top*). The levels of the Cdk9-CycT1 heterodimers and M.LARP7 proteins in WCEs (*Bottom*) and immunoprecipitates (*Middle*) were detected by Western blotting. WCEs were incubated with RNase A as indicated above the Western blots. (*D*) WCEs of HeLa cells that expressed the indicated M.BCDIN3 proteins were subjected to IP with the antibodies as shown (*Top*). The levels of the Cdk9-CycT1 heterodimers and M.BCDIN3 proteins in WCEs (*Bottom*) and immunoprecipitates (*Middle*) were detected by Western blotting. WCEs were incubated with RNase A as indicated above the Western blots.

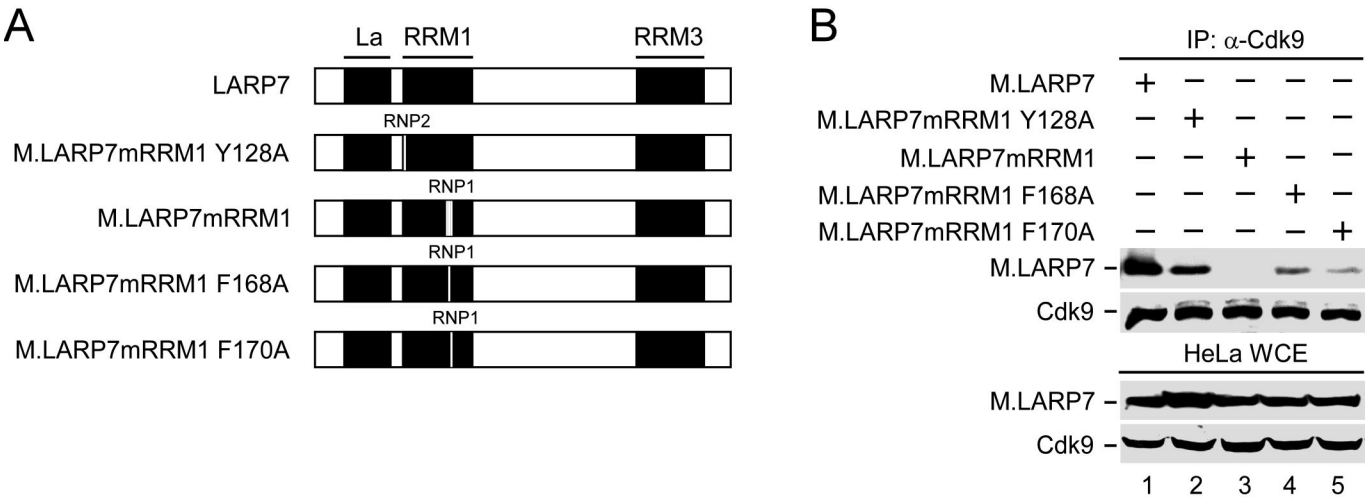

Fig. S3. Conserved amino acid residues within RNP1 in the LARP7 RRM1 domain are the most critical for the sequestration of LARP7 into 7SK snRNP. (*A*) Schematic representations of the wild-type LARP7 protein with its domains in black and the LARP7 mutant proteins containing point mutations in the RRM1 motif. (*B*) WCEs of HeLa cells that expressed the indicated Myc-tagged LARP7 (M.LARP7) proteins were subjected to IP with the α-Cdk9 antibody. The levels of Cdk9 and M.LARP7 proteins in WCE (*Bottom*) and immunoprecipitates (*Middle*) were detected by Western blotting.

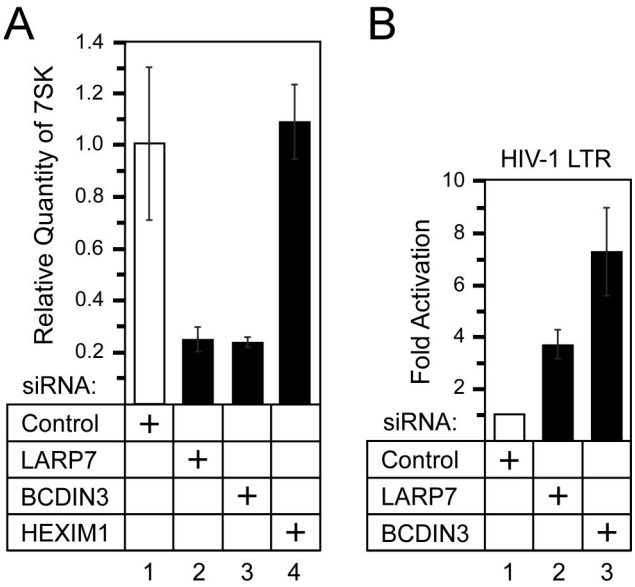

Fig. S4. Depletion of LARP7 or BCDIN3 decreases 7SK levels and activates P-TEFb-dependent HIV-1 transcription. (*A*) Relative quantities of 7SK levels were determined by RT-qPCR by using total RNA samples isolated from HeLa cells that expressed siRNAs as indicated below the graph. The error bars represent the mean ± SD. (*B*) HL3T1 cells containing an integrated HIV-1 LTR-driven luciferase reporter construct expressed the siRNAs as indicated below the graph. Luciferase activities in cell lysates were measured and the error bars represent the mean ± SD.

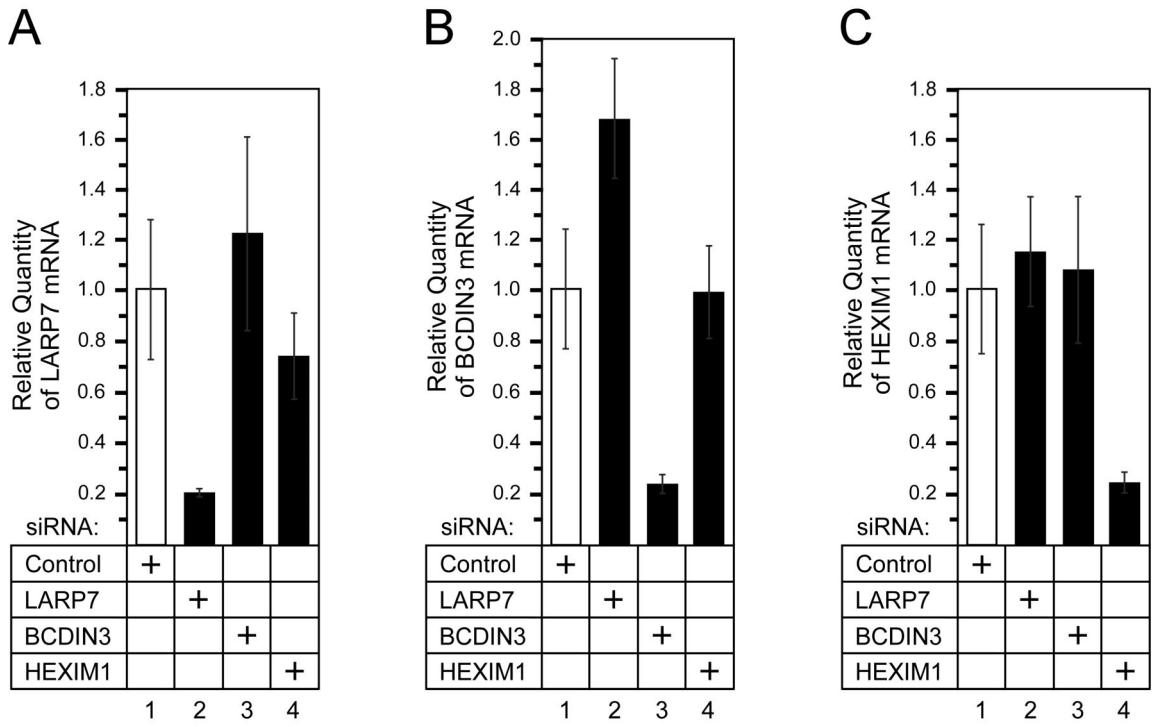

Fig. S5. Depletion of LARP7 protein levels on BCDIN3 siRNA expression occurs posttranscriptionally. (*A–C*) Relative quantities of LARP7, BCDIN3, and HEXIM1 mRNA levels were determined by RT-qPCR by using total RNA samples isolated from HeLa cells that expressed siRNAs as indicated below the graphs. The error bars represent the mean ± SD.

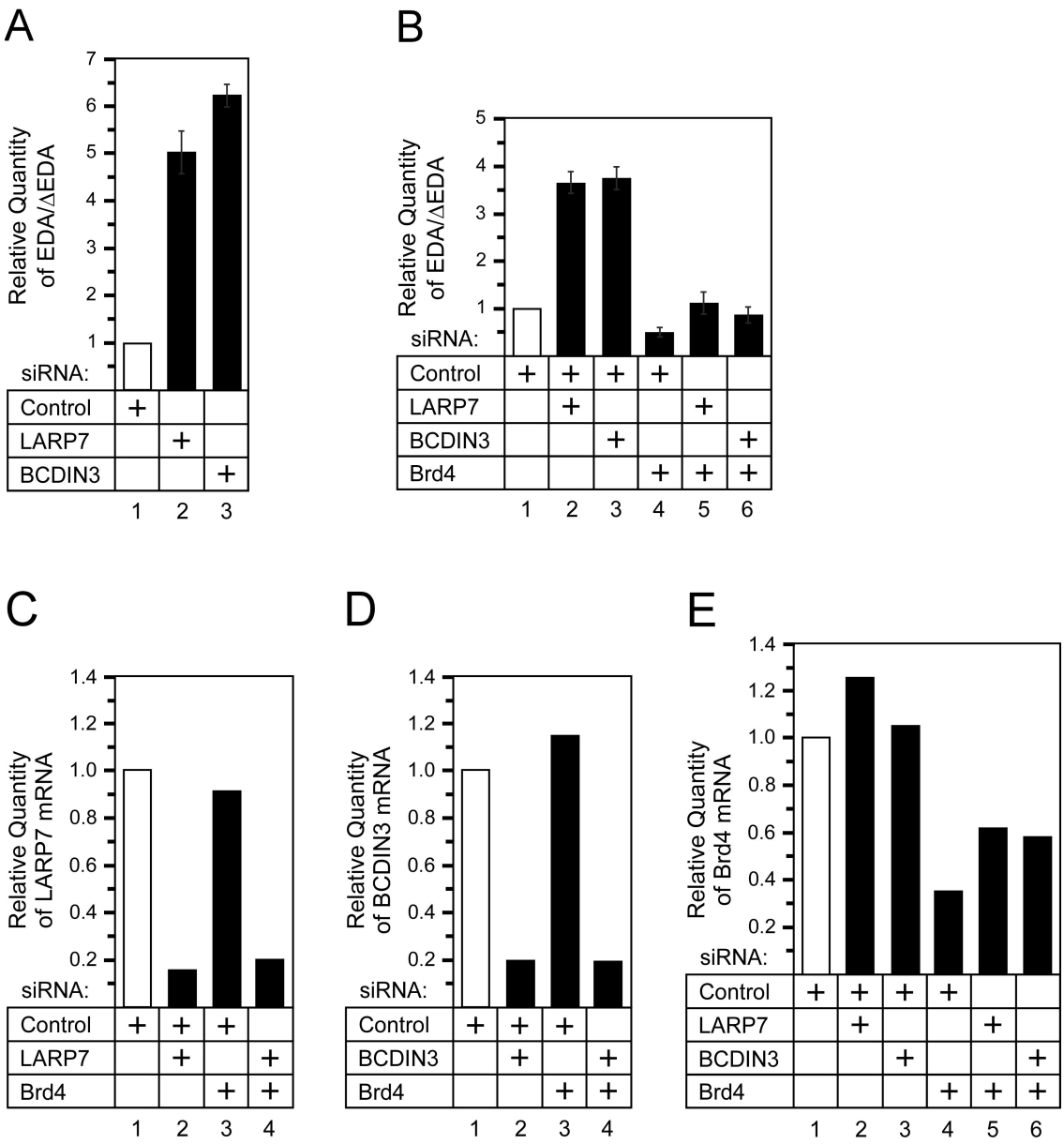

Fig. S6. Quantification of EDA inclusion on depleting LARP7 or BCDIN3. (*A* and *B*) Relative quantities of ΔEDA versus EDA mRNA ratios were determined by RT-qPCR by using total RNA samples isolated from HeLa cells that expressed siRNAs and pSVED-A Tot cassette as indicated below the graphs and employing the splicing variant-specific primer pairs. The error bars represent the mean ± SD. Knockdown efficiency of LARP7, BCDIN3, and Brd4 mRNAs (*C–E*) Relative quantities of LARP7, BCDIN3, and Brd4 mRNA levels were determined by RT-qPCR by using total RNA samples isolated from HeLa cells that expressed siRNAs as indicated below the graphs.

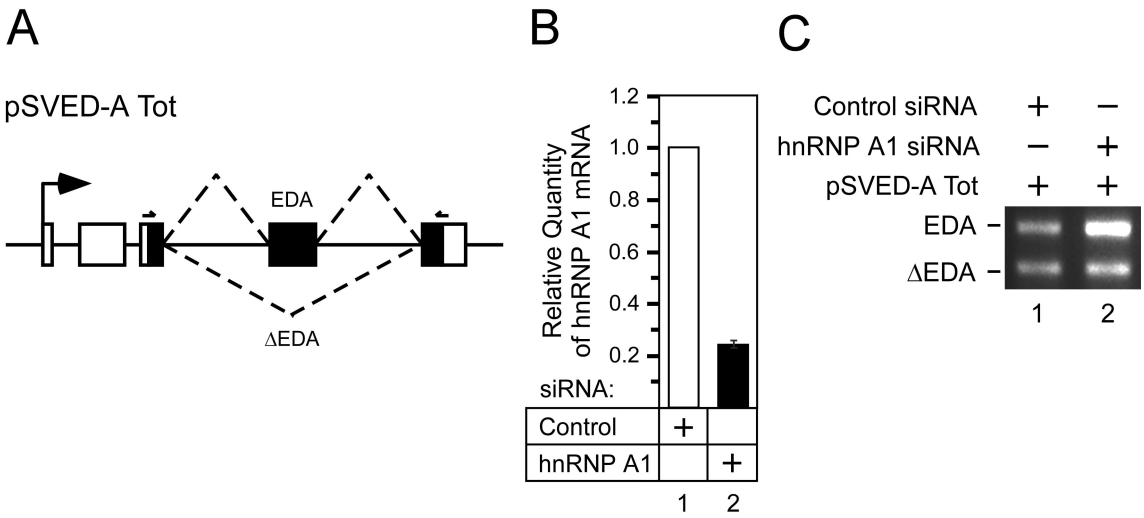

Fig. S7. Knockdown of hnRNP A1 enhances the inclusion of EDA. (*A*) Schematic representation of the pSVED-A Tot minigene cassette. Arrow represents the transcription start site from the α-globin promoter. The third exon of the 3 α-globin exons (white rectangles) contains 3 fibronectin exons (black rectangles; exon number 32–34) with the accompanying introns (solid lines). Dashed lines represent possible splicing patterns resulting in 2 mature EDA and ΔEDA mRNAs that include or lack the second fibronectin exon named EDA, respectively. Primer pair consisting of psv5′j and psv3′j that was used in RT-PCR analysis is indicated on top. (*B*) Relative quantity of hnRNP A1 mRNA levels were determined by RT-qPCR by using total RNA samples isolated from HeLa cells that expressed siRNAs as indicated below the graphs. The error bar represent the mean ± SD. (*C*) Levels of EDA and ΔEDA mRNAs were detected by RT-PCR by using total RNA samples isolated from HeLa cells that expressed siRNAs and pSVED-A Tot cassette as indicated above the agarose gels.

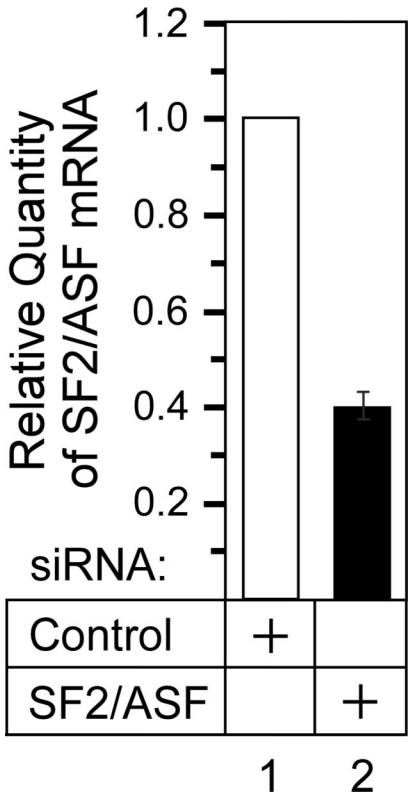

Fig. S8. Knockdown efficiency of SF2/ASF mRNA. Relative quantity of SF2/ASF mRNA levels were determined by RT-qPCR by using total RNA samples isolated from HeLa cells that expressed siRNA as indicated below the graphs. The error bar represents the mean ± SD.

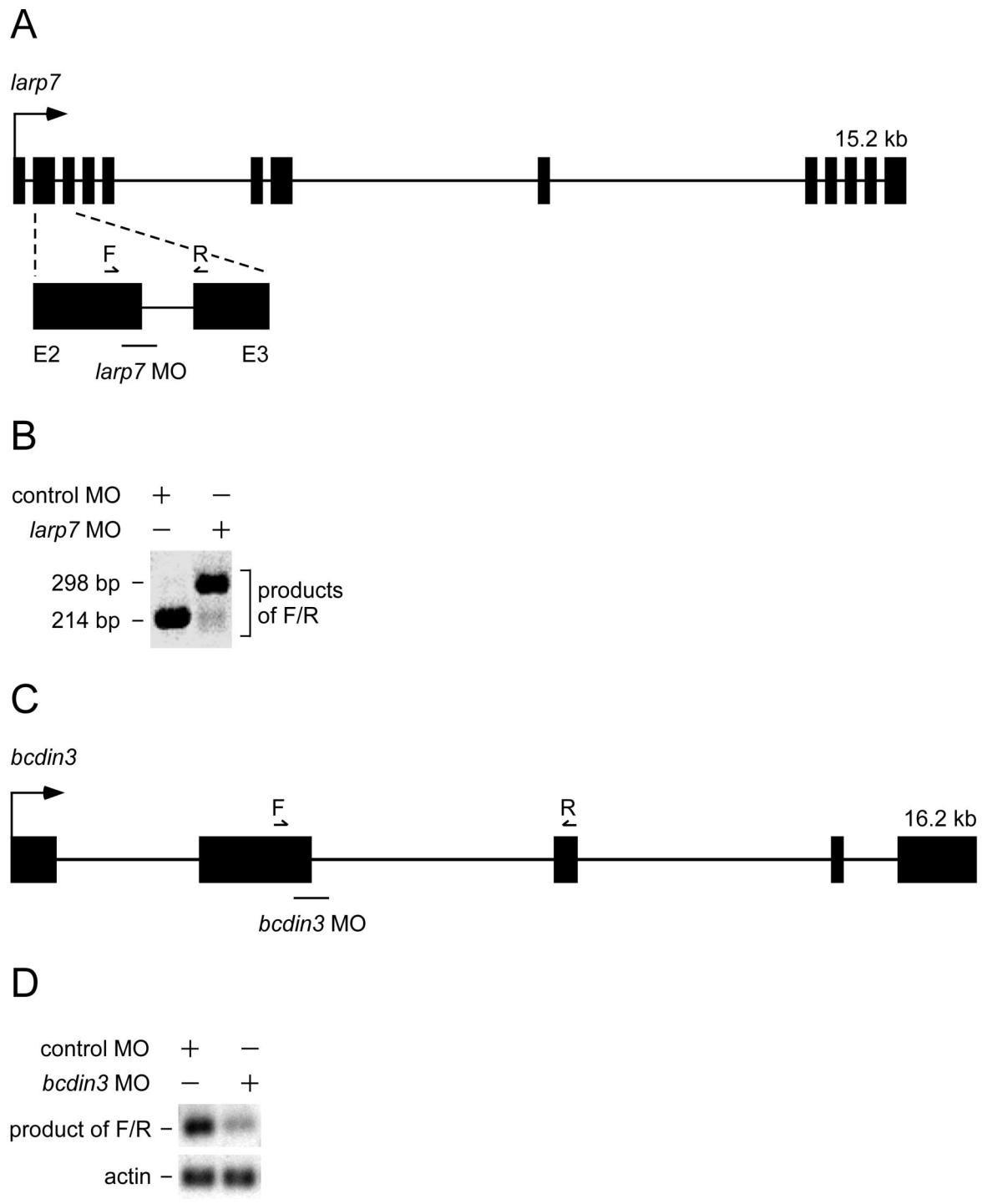

Fig. S9. Gene structure and efficacies of *larp7* and *bcdin3* MOs. (*A*) Schematic representation of the *D.rerio larp7* gene. Arrow represents the transcription start site. Exons are presented as black rectangles and introns as solid lines. The lower part of the schematic shows the enlarged region of the gene with exons 2 and 3 and the intron 2. The position of *larp7* MO is indicated below the schematic and the primers used for assessing the MO efficacy are indicated above. (*B*) Efficacy of *larp7* MO was determined by RT-PCR with the indicated primers in the schematic of *A* by using total RNA samples from control MO- or *larp7* MO-injected zebrafish embryos. (*C*) Schematic representation of the *D. rerio bcdin3* gene. Arrow represents the transcription start site. Exons are presented as black rectangles and introns as solid lines. The position of *bcdin3* MO is indicated below the schematic and the primers used for assessing the MO efficacy are indicated above. (*D*) Efficacy of *bcdin3* MO was determined by RT-PCR with the indicated primers in the schematic of panel *C* by using total RNA samples from control MO- or *bcdin3* MO-injected zebrafish embryos.

Other Supporting Information Files

Table S1
Table S2
Table S3
Table S4
Table S5
Table S6
Table S7