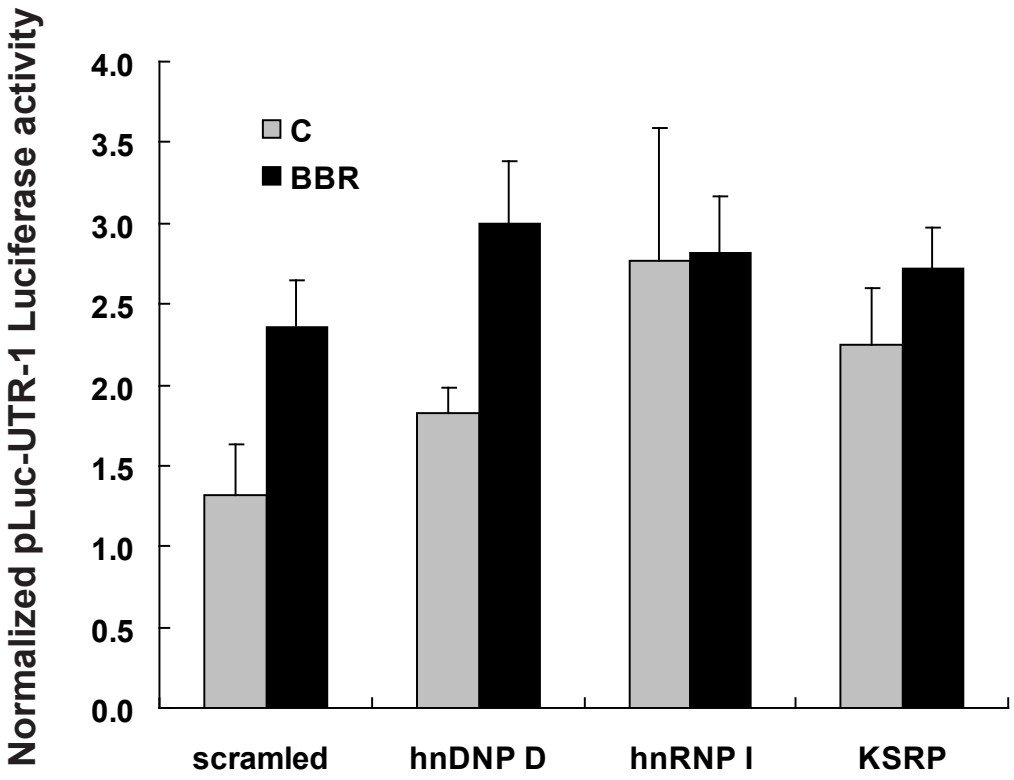
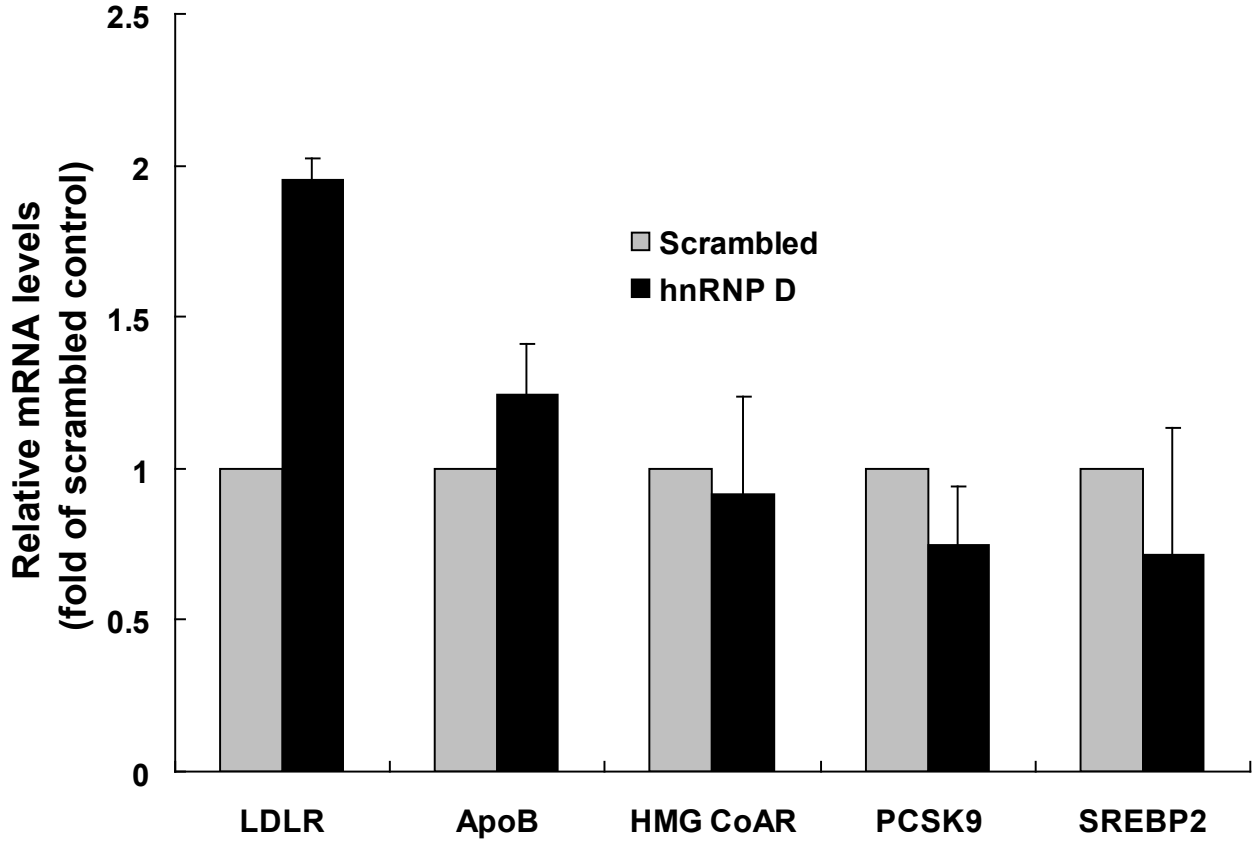
Supplemental Figure 1

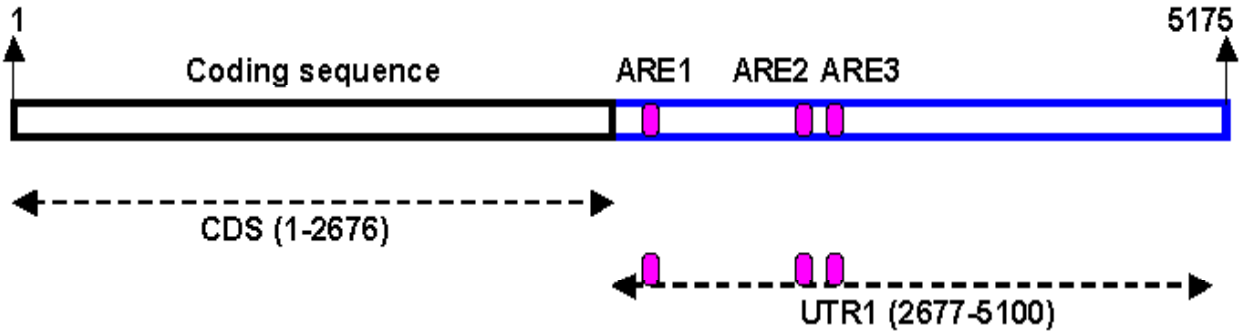


Supplemental Figure 2



Supplemental Figure 3

A



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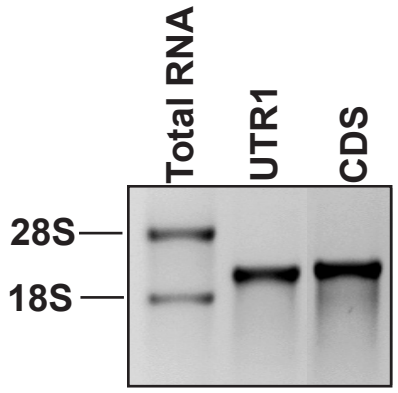

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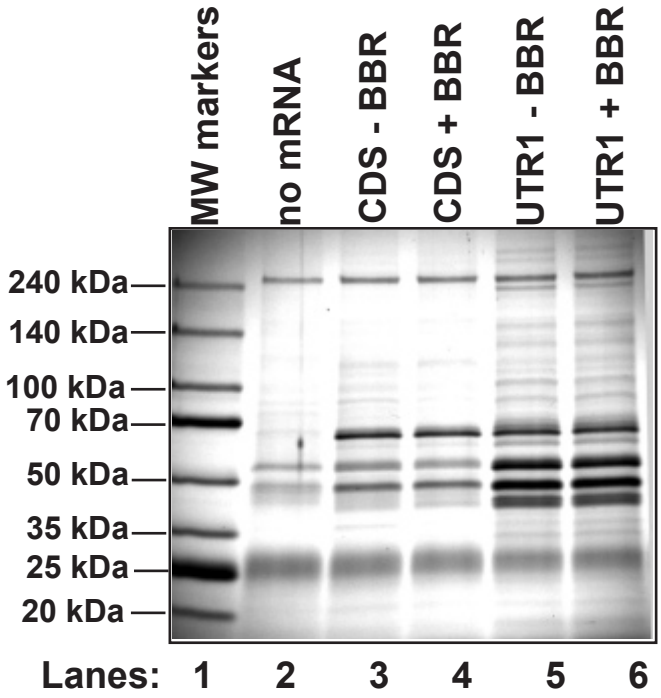
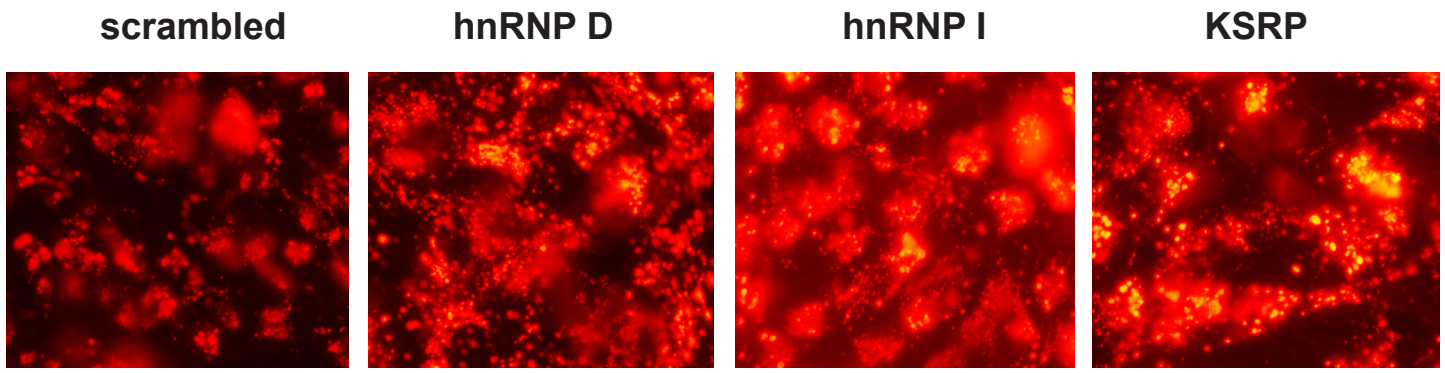
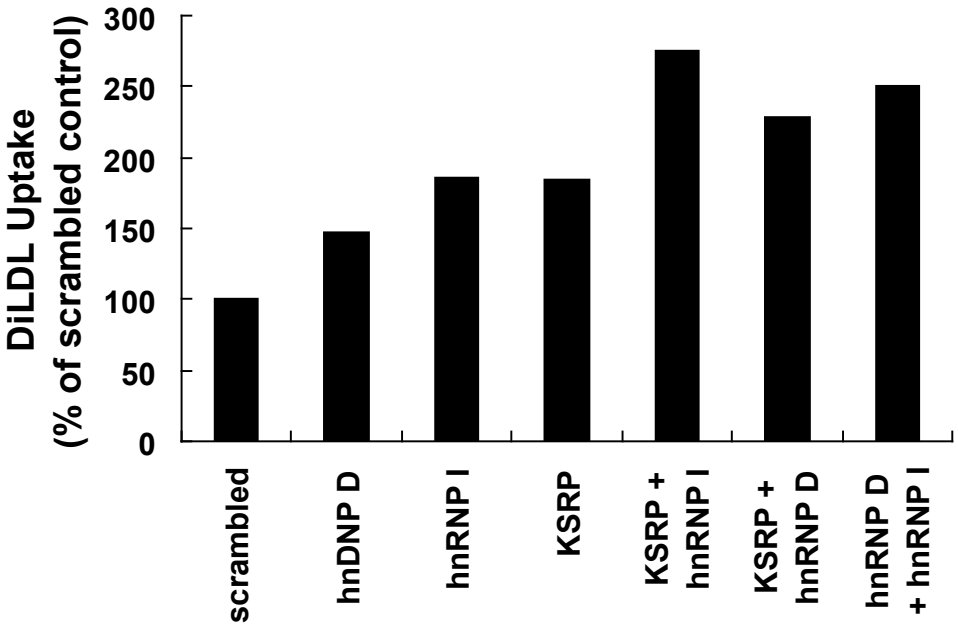


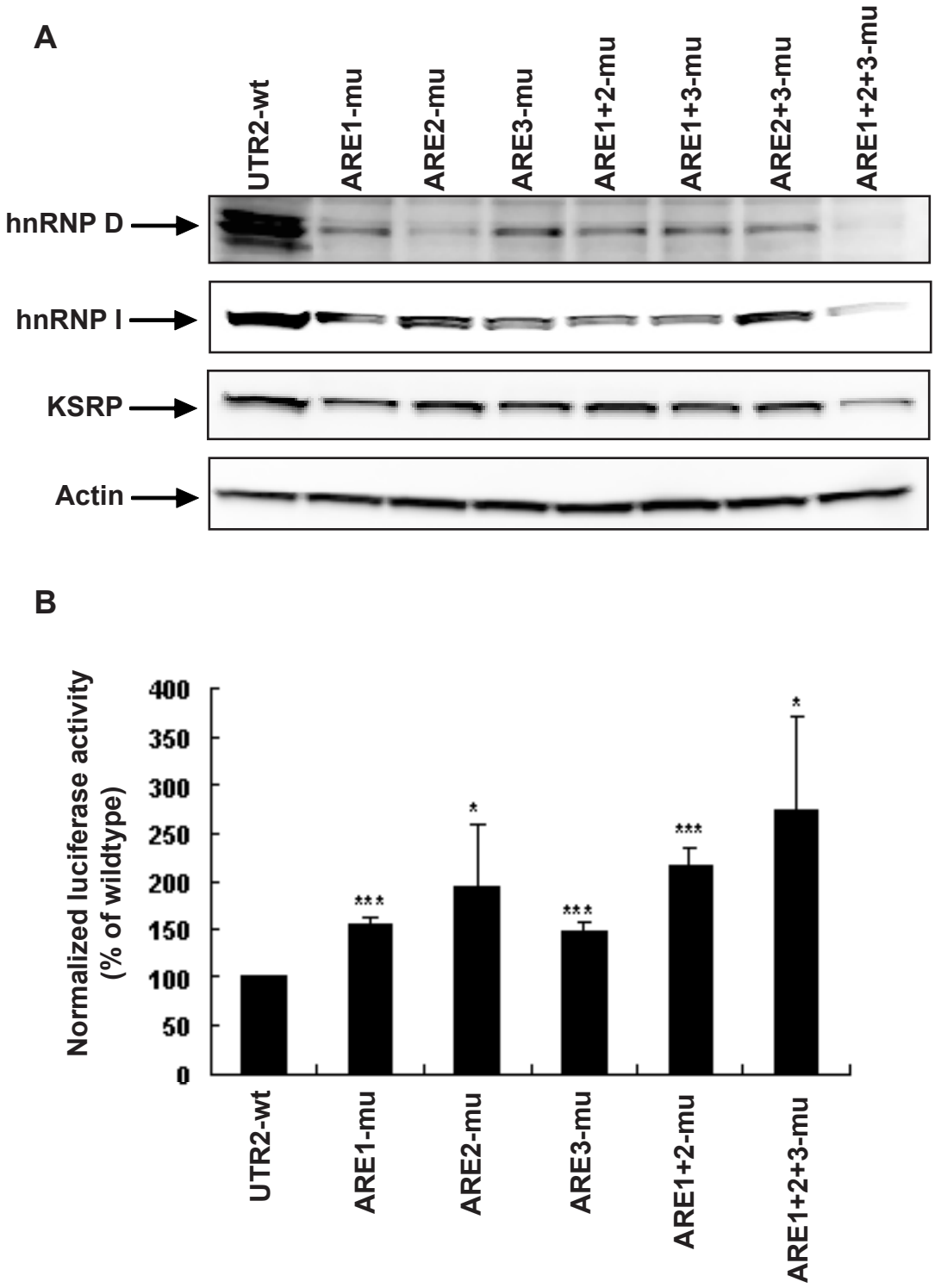
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure Legends

Figure 1. Analysis of effects of siRNAs targeted to hnRNP D, hnRNP I, and KSRP on pLuc-UTR-1 activity by transient transfection assays. HepG2 cells ($1x10^4$ cells/well) in suspension were mixed with 12 nM siRNA in the Silencer™ siRNA transfection reagent and were plated in 96 well plates. Twenty-four hours later, cells were transfected with plasmid DNA of pLuc-UTR-1 and the control plasmid pRL-SV40 at the ratio of 100:1 using FuGene 6 transfection reagent. Twenty-four hour post transfection, the medium was changed to 0.5% FBS-MEM and cells were treated with BBR at a dose of 15 μg/ml or the BBR carrier DMSO (0.1%) for 8 h prior to cell lysis. Activities of firefly luciferase and renilla luciferase in cell lysate were measured using the Dual Luciferase Reporter Assay System (Promega). Triplicate wells were assayed for each condition. The graph shown is a representative of 3 separate transfection experiments.

Figure 2. Transfection of hnRNP D siRNA increased LDLR mRNA abundance without affecting mRNA levels of other genes involving in LDL-cholesterol metabolism. siRNAs were transfected into HepG2 cells in a 6-well plate and total RNA was isolated after two days of transfection. Real-time RT-PCR analysis was conducted with predesigned TagMan probes for LDLR, ApoB, HMGCR, PCSK9, and GAPDH for normalization. Real-time RT-PCR analysis of SREBP2 and actin were conducted with SYBR Green 1 and gene specific primers. After normalization with GADPH or actin, the fold changes were calculated by dividing mRNA levels in hnRNP D siRNA transfected cells with mRNA levels in scrambled siRNA transfected cells.

Figure 3. Biotinylated LDLR transcript pull-down assays.

(A) Schematic of LDLR mRNA and the biotinylated RNA fragments corresponding to the coding sequence (CDS) or to the 3'UTR sequence (UTR1).

(B) In vitro transcribed and biotinylated CDS and UTR1 of LDLR mRNA. CDS or UTR1 region of LDLR gene was PCR cloned into pcDNA 3.1(+) and was in vitro transcribed in the presence of biotin-16-UTP by using AmpliScribe™T7-Flash™ transcription Kit.

(C) 500 μg of cytoplasmic protein prepared from control or BBR-treated cells were incubated with 30 μg of biotinylated CDS or UTR1. The formed mRNA-protein complexes were isolated through NeutrAvidin™ Agarose Resin. Proteins bound to biotinylated RNA were subsequently eluted and separated by SDS gel. Protein bands were visualized by blue dye staining. Lane 1 shows molecular weight marker and lane 2 is a control sample lacking biotinylated mRNA.

Figure 4. Effects of siRNA knockdown on LDLR-mediated uptake of DiI-LDL particles. HepG2 cells were seeded onto 6-well plates and transfected with siRNAs for 48 h. Cells were incubated in MEM containing 0.5% FBS overnight prior to the addition of DiI-LDL (5 μg/ml). After 4 h, the uptake of DiI-LDL was examined with a fluorescent microscope and was analyzed by FACS. The mean fluorescence value (MFV) of control cells transfected with scrambled siRNA was defined as 100% and the MFV in specific siRNA transfected cells were plotted relative to that value. The data shown are the average of two independent experiments.

Figure 5. Effects of siRNA knockdown individually and combined on LDLR-mediated uptake of DiI-LDL particles. HepG2 cells were seeded onto 6-well plates and transfected with individual siRNAs or two siRNAs. MFV was determined as described in Fig. 4 legend.

Figure 6. Effects of ARE mutations individually and combined on the binding of ARE-BPs to LDLR 3'UTR transcripts and on luciferase-LDLR 3'UTR reporter activities.

(A) Biotinylated RNA pull-down assays were carried out by incubating 100 μg of cytoplasmic protein with 5 μg of each biotinylated transcript. Bound proteins in the pull-down material were analyzed by western blotting using antibodies recognizing hnRNP D, I, and KSRP. The abundance of β-actin in the supernatant from each sample was analyzed by western blotting to demonstrate the equal protein loading. The graph shown is a representative of 3 separate pull-down assays.

(B) HepG2 cells were transiently transfected with individual luciferase-UTR reporter plasmids along with pRL-SV40 as the normalizing transfection vector. Cells were harvested two days after transfection and dual reporter assays were performed. The normalized firefly luciferase activity in cells transfected with the wt vector was defined as 100%, and luciferase activities in cells transfected with mutated vectors were plotted relative to that value. The data represent means ± SD derived from 3 independent transfection experiments in which triplicate were assayed for each condition. *$p < 0.05$ and ***$p < 0.001$ as compared to UTR-wt.