Presomitic mesoderm-specific expression of the transcriptional repressor *Hes7* is controlled by E-box, T-box, and Notch signaling pathway

Shinichi Hayashi[1,3], Yasukazu Nakahata[1]*, Kenji Kohno[2], Takaaki Matsui[1], Yasumasa Bessho[1]*

SUPPLEMENTAL EXPERIMENTAL PROCEDURES and SUPPLEMENTAL FIGURES (S1-S6) are included.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Bisulfite Sequence

Genome sample was obtained from E10.5 or adult mouse tissue and digested by *EcoR*I. Sample was treated with 0.3 N NaOH at 37 °C for 20min, and then it was reacted with 3 M Sodium Bisulfite and 0.5 mM Hydroquinone at 55 °C for 16 hr. After DNA purification, DNA samples were treated with 0.3 N NaOH at 37 °C for 20 min and purified again. Sample were amplified by PCR and cloned for sequence analyses. The sequences of the forward and reverse primers for *Hes7* and *Olig1* are as follows; *Hes7* FW: 5'-ATT ATT GAA TTA TTA ATG TAG ATA AGG AGA GAG-3', *Hes7* RV: 5'-AAA TAA CTA TAA AAT CAA AAA CAA AAT AA AA CC-3', *Olig1* FW: 5'-GAG AGA GTT TAT TTG GGT GAA GAT AAG AG-3', *Olig1* RV: 5'-AAT AAT CTC TCT CTA ACT TTC TTC TCT ACC-3'.

OligoDNA pull-down assay

See "EXPERIMENTAL PROCEDURES" for sample preparations. OligoDNA sequences are as

follows; T-box2 FW: 5'-AGG GGC GGC CCC ACA CCC GGG TGC AAA-3', T-box2 RV: 5'-TTT GCA CCC GGG TGT GGG GCC GCC CCT-3', T-box2 mut FW: 5'-AGG GGC GGC CCg AtA tCC GGG TGC AAA-3', T-box2 mut RV: 5'-TTT GCA CCC GGa TaT gGG GCC GCC CCT-3'. Smal lettters indicate mutated nucleotides.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed using reagents provided in the LightShift Chemiluminescent EMSA kit (Thermo Scientific, USA). Briefly, HEK293T cells were seeded in 10 cm dish (3 x 10^6 cells/dish) and transfected with 4 μg of expression vectors and cultured for 48 hr. Cells were lysed in TNE buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 1% Nonidet P40, 150 mM NaCl) with proteinase inhibitor cocktail (Nacalai tesque, Japan). Cell extracts containing the indicated protein were pre-incubated with 4 pmol of non-labeled DNA and 1 μg poly (dI-dC) in binding buffer for 10 min, and then 20 fmol biotin 5’ end-labeled DNA was added to the reaction mixture and incubated for 30 min. Binding reactions were resolved on a polyacrylamide gel and electrophoretically transferred to nylon membrane. The transferred DNA was crosslinked to membrane using a transilluminator, FUNA-UVLINKER FS-1500 (Funakoshi, Japan). Biotin-labeled DNA was detected by chemiluminescence and exposed to X-ray film. OligoDNA sequences are as follows; T-box3 FW: 5'-CCT GCT GGG ACC ACA CAT CTG TGC TTC-3', T-box3 RV: 5'-GAA GCA CAG ATG TGT GGT CCC AGC AGG-3', E-box2&3 mut FW: 5'-CCC GAG CCT gcC GgG ggG GgG AGA AAA ACT-3', E-box2&3 mut RV: 5'-AGT TTT TCT CcC Cgc CcC Ggc AGG CTC GGG-3'. Smal lettters indicate mutated nucleotides. See "OligoDNA pull-down assay" section in the EXPERIMENTAL

PROCEDURES for other OligoDNA sequences.

Figure S1

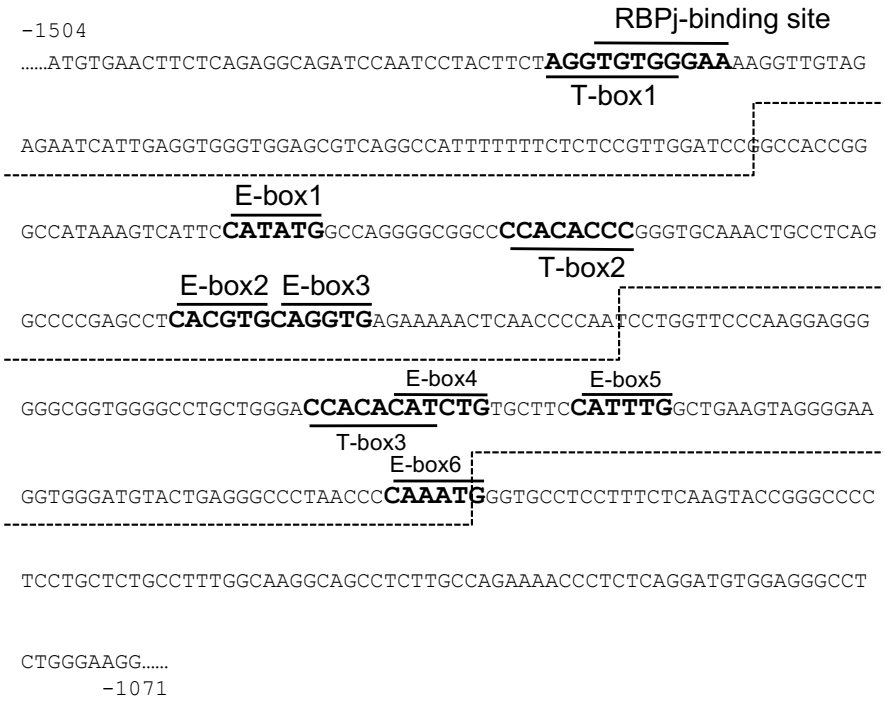

Figure S1. Sequence of mouse *Hes7* C region.

Putative T-boxes, E-boxes and RBPjk binding site within mouse *Hes7* C region. Dotted lines separate regions corresponding to Deletion1-4.

Figure S2

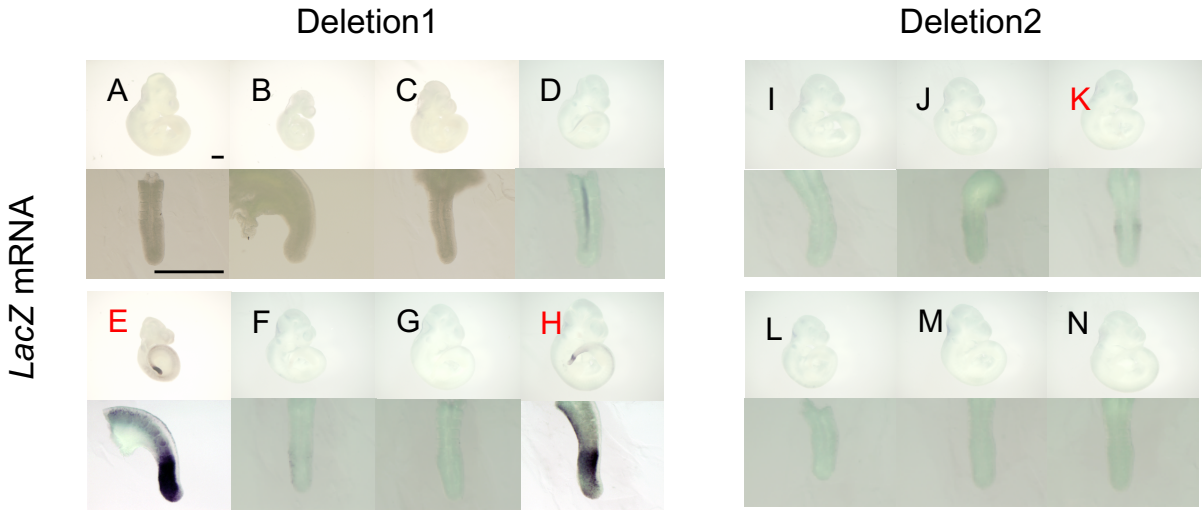

Figure S2. *LacZ* mRNA distribution by Deletion mutant reporters in transgenic mice.
Transgenic mice integrated with Deletion mutant reporters were stained with *LacZ* probe at E10.5 by *in situ* hybridization. (A-H) 2 embryos (E, H) out of 8 showed expressions by Deletion1 reporter. (I-N) 1 embryo (K) out of 6 showed an obscure expression by Deletion2 reporter. Scale bars, 1mm.

Figure S3

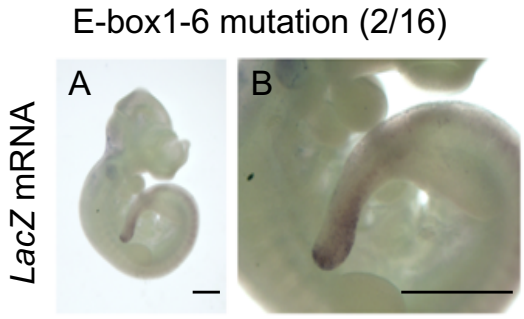

Figure S3. *LacZ* mRNA distribution by E-box1-6 mutant reporter in transgenic mice.
Transgenic mice integrated with E-box1-6 mutant reporter were stained with *LacZ* probe at E10.5 by in situ hybridization. (A) 14 embryos out of 16 did not show any signal, but 2 out of 16 showed an obscure expression. (B) High magnification image of A. Scale bars, 1mm.

Figure S4

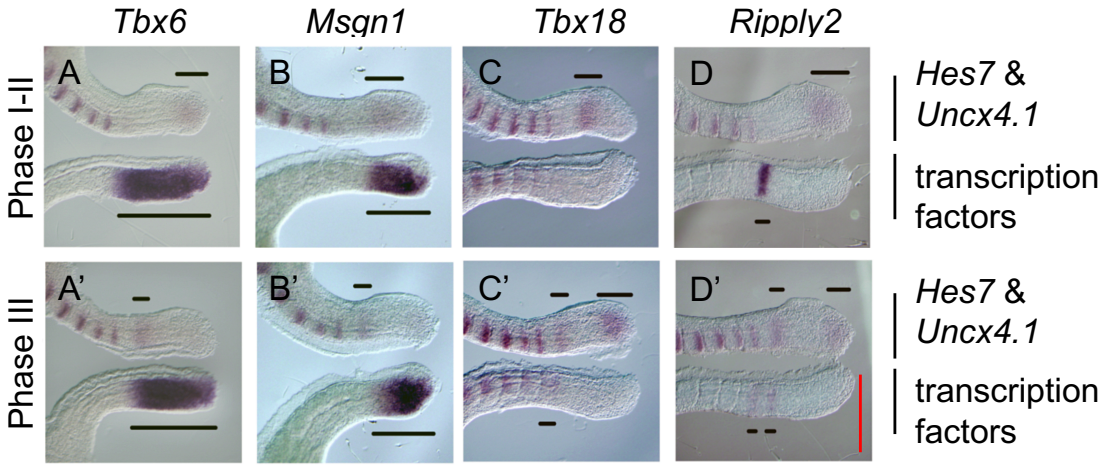

Figure S4. Comparison of expression domain between *Hes7* and its regulators.

PSMs at E10.5 were bisected into two along neural tube and stained by *in situ* hybridization. Bottom halves were detected transcription factors and upper halves were detected *Hes7* transcribed region. (A, A') *Tbx6* mRNA was distributed ubiquitously in the PSM besides the anterior-most region. Each phase of *Hes7* overlapped with Tbx6 expression domain. (B, B') *Msgn1* mRNA was expressed in the posterior PSM. Although *Hes7* transcribed region in phase III did not coincide with *Msgn1* expression domain, a main part of the transcribed region overlapped with *Msgn1*. Expression pattern of *Tbx6* and *Msgn1* was constant during *Hes7* propagation. (C, C') *Tbx18* was expressed in the rostral somites and the anterior PSM. (D, D') *Ripply2* showed a striped shape pattern in the anterior PSM. Black bars indicate expression domain of transcription factors. Scale bar (vertical red bar), 0.5mm.

Figure S5

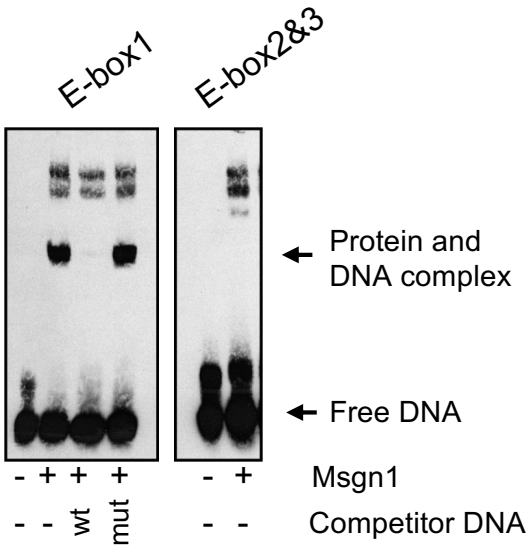

Figure S5. Msgn1 binds to E-box1, but not E-box2&3.

EMSA was performed using 20 fmol of biotin 5' end-labeled E-box elements indicated with Msgn1-Flag. Competitor DNAs were 4 pmol of wild type (wt) or mutant (mut) non-labeled E-box1.

Figure S6

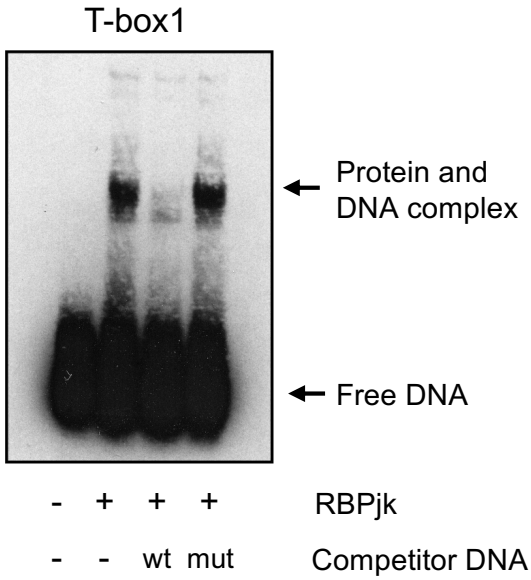

Figure S6. RBPjk binding to *Hes7* promoter region *in vitro*.

EMSA was performed using 20 fmol of biotin 5' end-labeled T-box1 with RBPjk-Flag. Competitor DNAs were 4 pmol of wild type (wt) or mutant (mut) non-labeled T-box1.

Figure S7

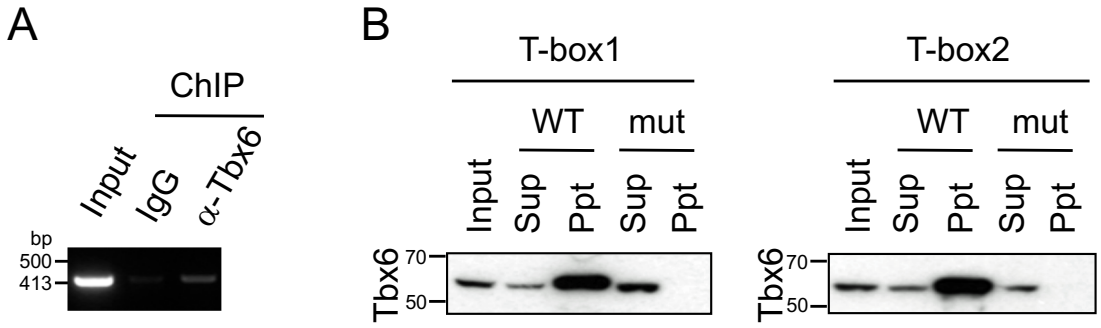

Figure S7. Tbx6 binding to *Hes7* promoter region *in vitro* and *in vivo*.

(A) ChIP assay with anti-Tbx6 antibody or normal rabbit IgG (negative control) were performed using mice PSM. Resultant genomic fragment was amplified using *Hes7* C region specific primer. (B) OligoDNA pull-down assays against wild type or mutant T-box1 and T-box2 sequences with Tbx6-Flag were performed. Tbx6 was revealed with anti-Flag.

Figure S8

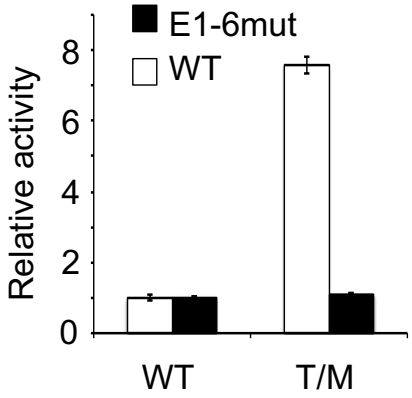

Figure S8. Msgn1 is essential for the synergistic effect with Tbx6.

Luciferase assays for wild type or E-box1-6 mutant *Hes7* C region with Tbx6 and Msgn1 (T/M) were performed. Reporter firefly luciferase activities were normalized by Renilla luciferase activities under SV40 promoter. Data represent the means ± SEM of three independent samples.

Figure S9

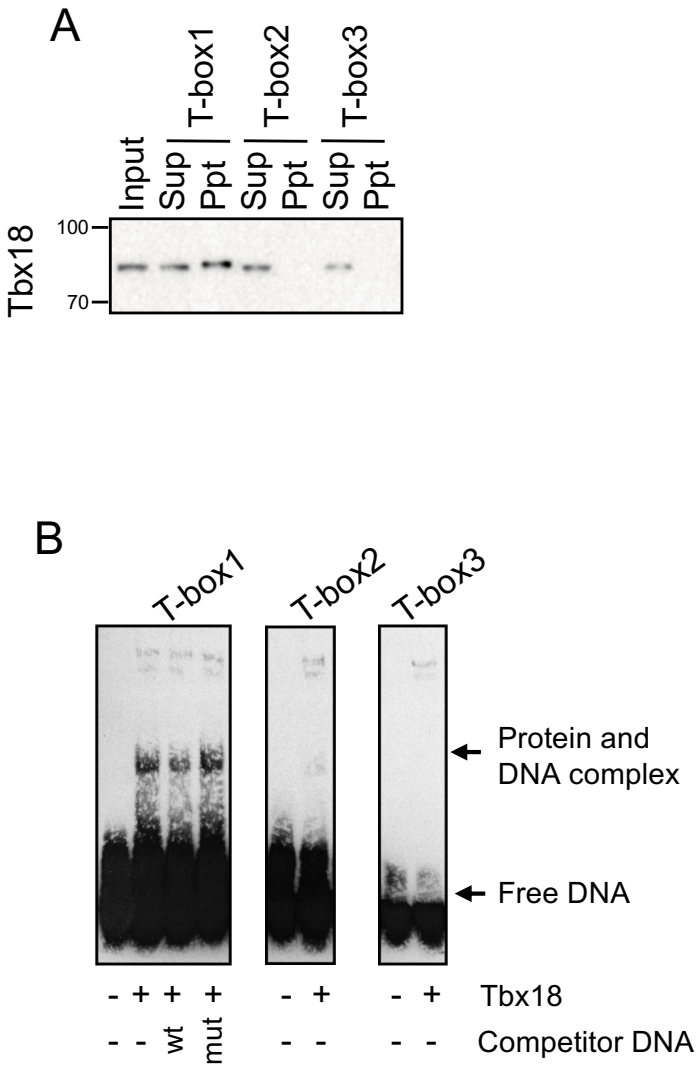

Figure S9. Tbx18 binds to T-box1, but not to T-box2 or 3.

(A) OligoDNA pull-down assays against wild type T-box1, 2 or 3 with Tbx18-Flag were performed. Tbx18 was revealed with anti-Flag. (B) EMSA was performed using 20 fmol of biotin 5' end-labeled T-box elements indicated with Tbx18-Flag. Competitor DNAs were 4 pmol of wild type (wt) or mutant (mut) non-labeled T-box1.

Figure S10

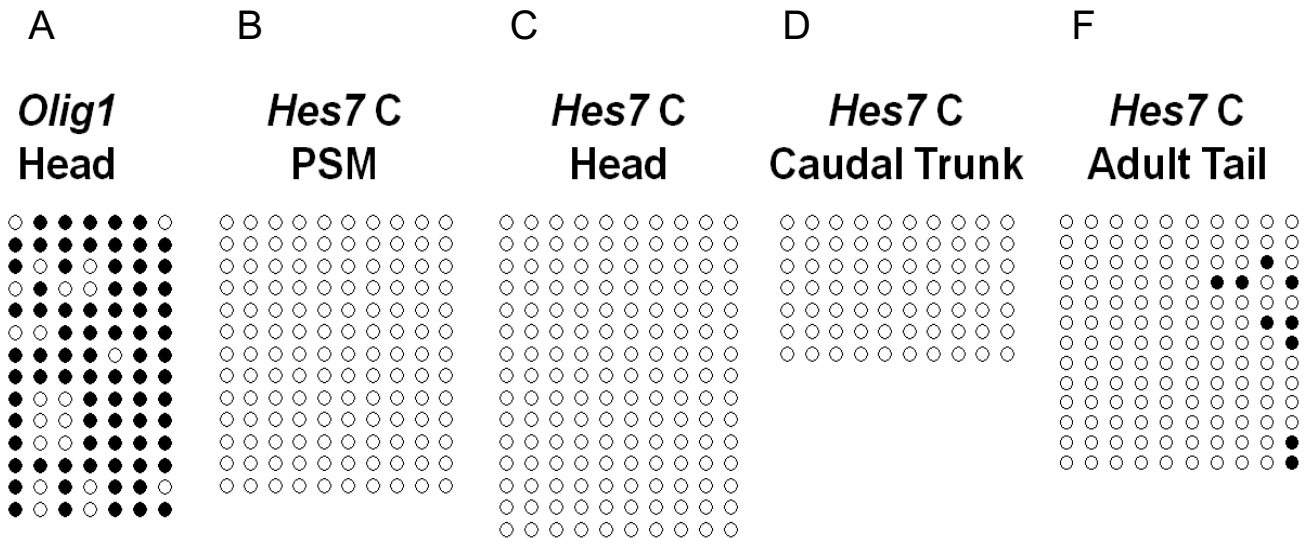

Figure S10. Methylation status of *Hes7* C region.

(A) Methylation status of *Olig1* promoter from -3726 to -3341. Genomic DNA was collected from Head at E10.5 and subjected to bisulfite sequencing as a control. (B-F) Methylation status of *Hes7* C region. Genomic DNA was obtained from the PSM (B), the Head (C), the caudal trunk (D) at E10.5 and Tail at adult (F), and subjected to bisulfite sequencing. Methylated and unmethylated CpG sites were indicated as black circle and white circle, respectively.