

Fig. S1. sgRNA positions on and around the *Mesp1*/*Mesp2* locus

(A) sgRNA and primer positions on *Mesp1* and *Mesp2* genes. Mesp1-1, Mesp1-5, Mesp2-2, and Mesp2-5 were used for F0 assay and KO lines with a small indel. Mesp1-5' and Mesp1-3'-2 were used to establish *Mesp1*ΔNeo mice. Mesp1-1 and Mesp1-3' were used to establish *Mesp1*Δ573 and *Mesp1*Lefty2 knock-in. Mesp2-1, Mesp2-5, and Mesp2-6 were used to make F0 *Mesp1*Lefty2; *Mesp2* KO.

(B) sgRNA and primer positions around the *Mesp1* early mesoderm enhancer (EME). The EME is shown with a white box and the highly conserved region2 (HCR2) is shown with a gray box. Schematic representation of each enhancer deletion line is shown below.

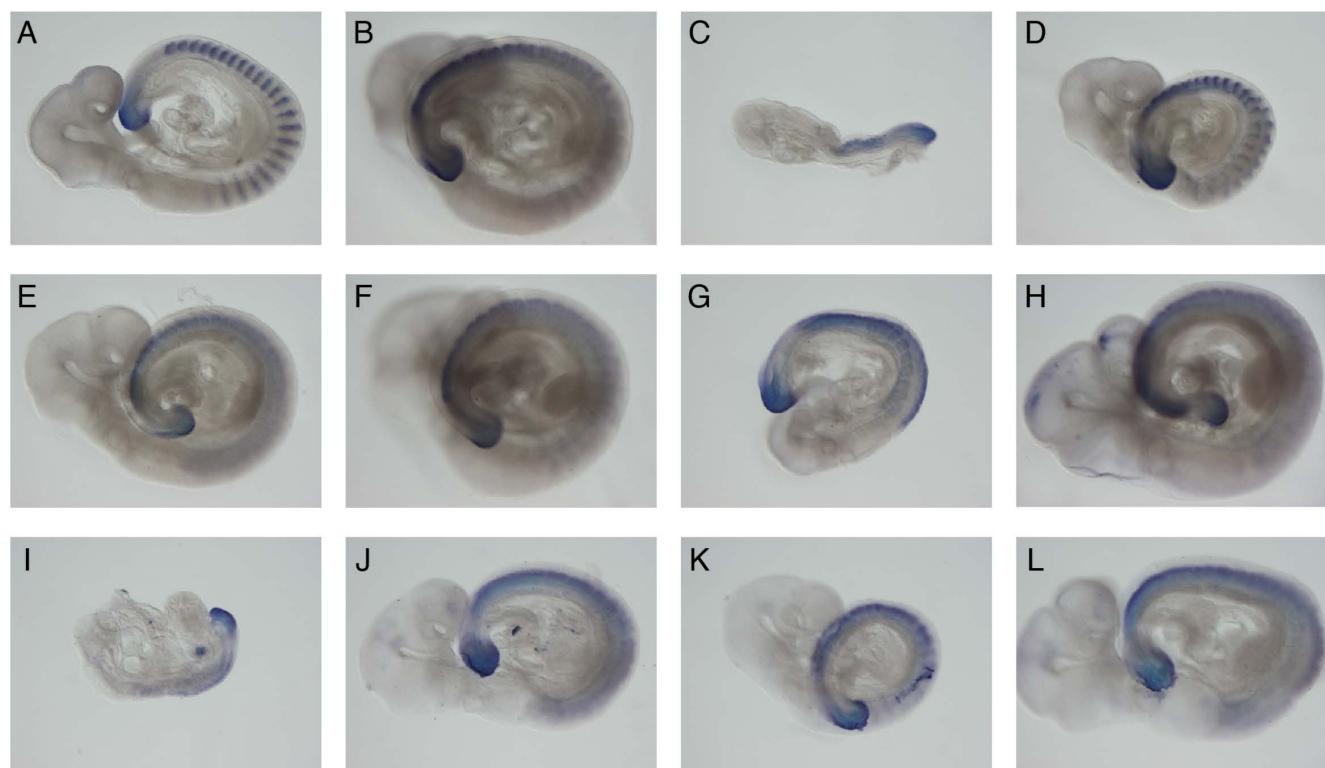


Fig. S2 Variation in the somitogenesis phenotype among Type II F0 *Mesp1/Mesp2* mutant embryos Embryos obtained by F0 assay were subjected to whole-mount in situ hybridization with *Uncx4.1* (caudal somite marker) and *T* (presomitic mesoderm marker) probes.

(A) WT (B-F) and Type II F0 *Mesp1/Mesp2* mutant embryos with Mesp1-1 and Mesp2-1 sgRNAs.

(G-L) Type II F0 *Mesp1/Mesp2* mutant embryos with Mesp1-5 and Mesp2-5 sgRNAs.

Genotyping results of each embryo are listed in Table S1.

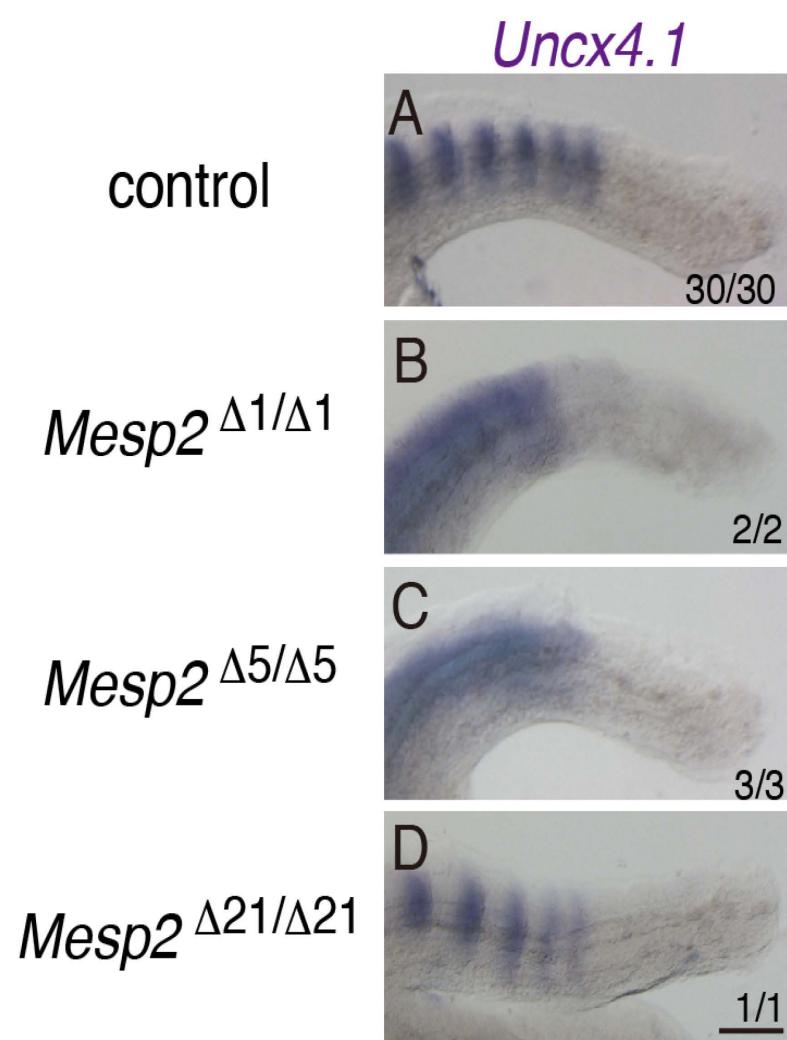
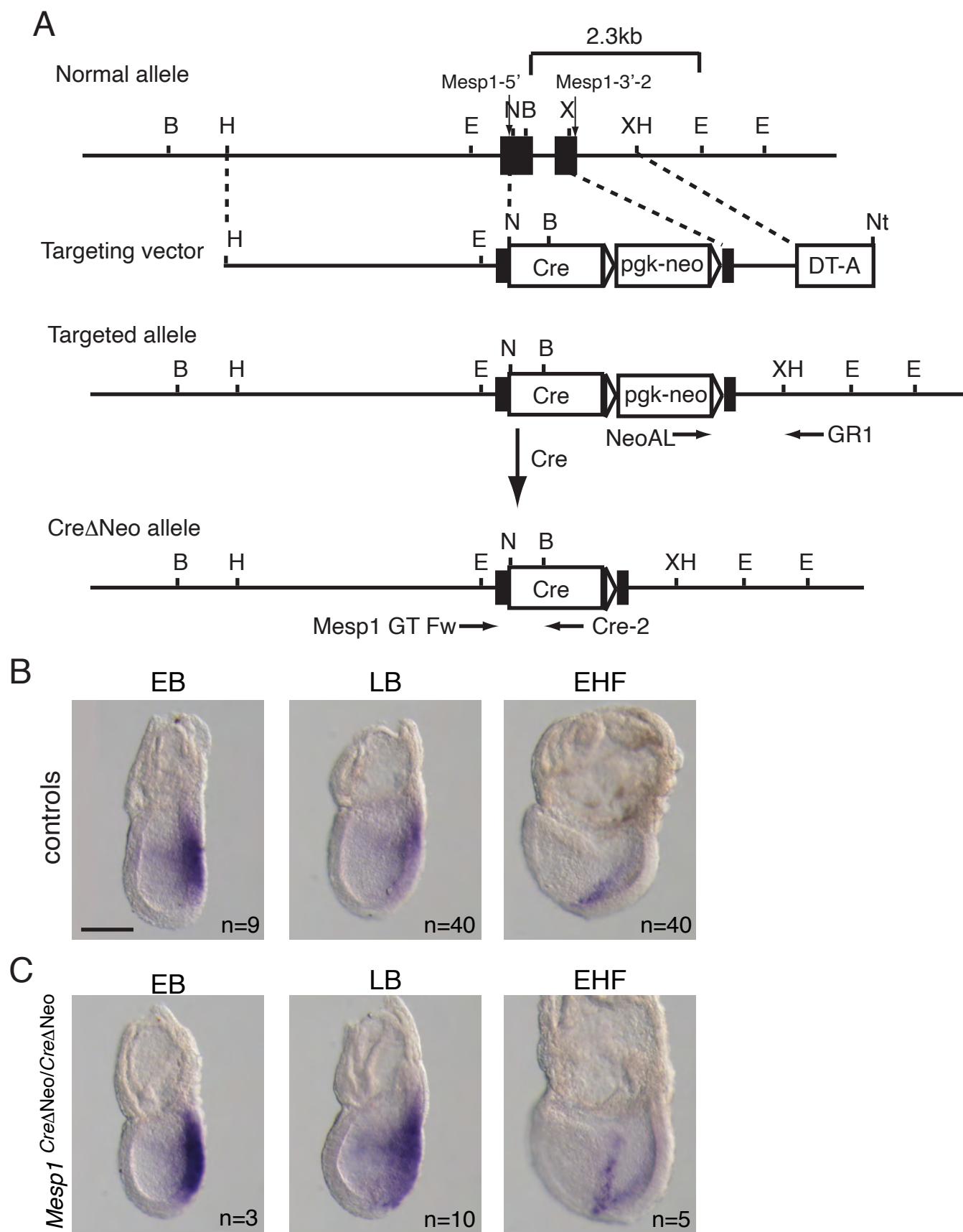
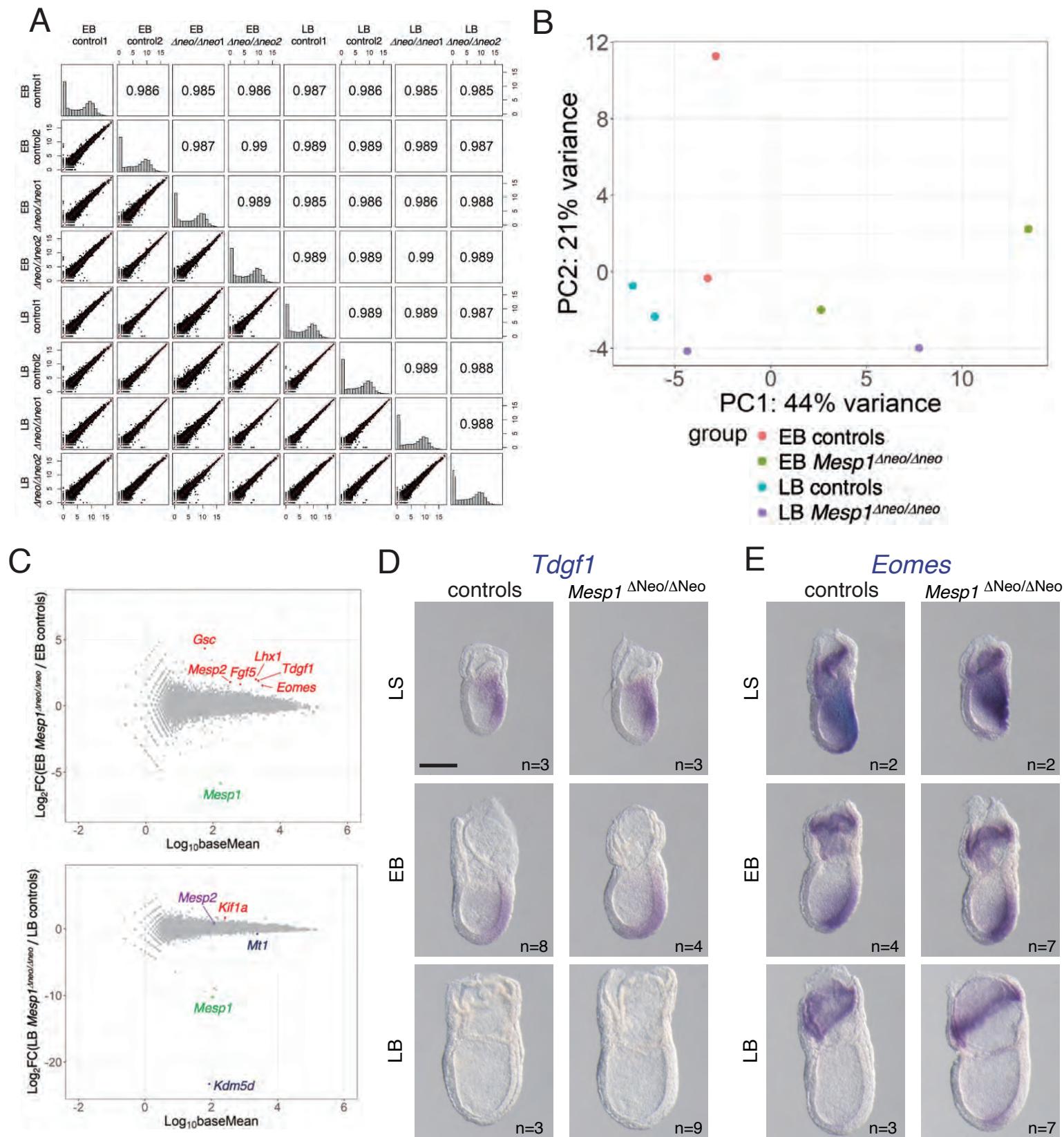


Fig. S3 CRISPR/Cas9-mediated *Mesp2* KO embryos reproduced the caudalized somite phenotype.
(A-D) Whole-mount *in situ* hybridization of indicated genotype embryos with the *Uncx4.1* probe. Numbers indicate the examined embryos with the same phenotypes. Scale bar indicates 200 μ m.

**Fig. S4. Establishment of the *Mesp1Cre Δ Neo* mouse line.**

(A) Schematic representation of the targeting strategy for the *Mesp1Cre Δ Neo* mouse line. Restriction sites: B, BamHI; E, EcoRI; H, HindIII; N, Ncol. (B, C) Whole-mount *in situ* hybridization of controls: WT and heterozygous (B), and *Mesp1Cre Δ Neo/Cre Δ Neo* embryos (C) at E7.5 using the *Mesp2* probe. Staging was determined based on Downs and Davies 1993. Scale bar indicates 200 μ m. Numbers indicate examined embryos.

**Fig. S5. RNA seq analysis of the controls and *Mesp1*^{ΔNeo/ΔNeo} embryos.**

(A) Scatterplot matrix representing pairwise scatterplots (below diagonal) and pairwise Pearson correlation coefficients (r , above diagonal).

(B) Principal component analysis of sequenced samples.

(C) MA plot analysis of the EB stage and LB stage embryos. Genes used for analysis are shown in gray. Red indicates significantly upregulated genes, and Blue indicates significantly downregulated genes ($\text{padj} < 0.01$). *Mesp1* is indicated in green, and *Mesp2* in the LB stage plot is indicated in Purple. (D, E) Whole-mount in situ hybridization of controls and *Mesp1*^{ΔNeo/ΔNeo} embryos at E7.5 using the *Tdgif1*(D) and *Eomes* (E) probes. Staging was determined based on Downs and Davies 1993. Scale bar indicates 200 μm . Numbers indicate examined embryos.

Supplemental Figure 6

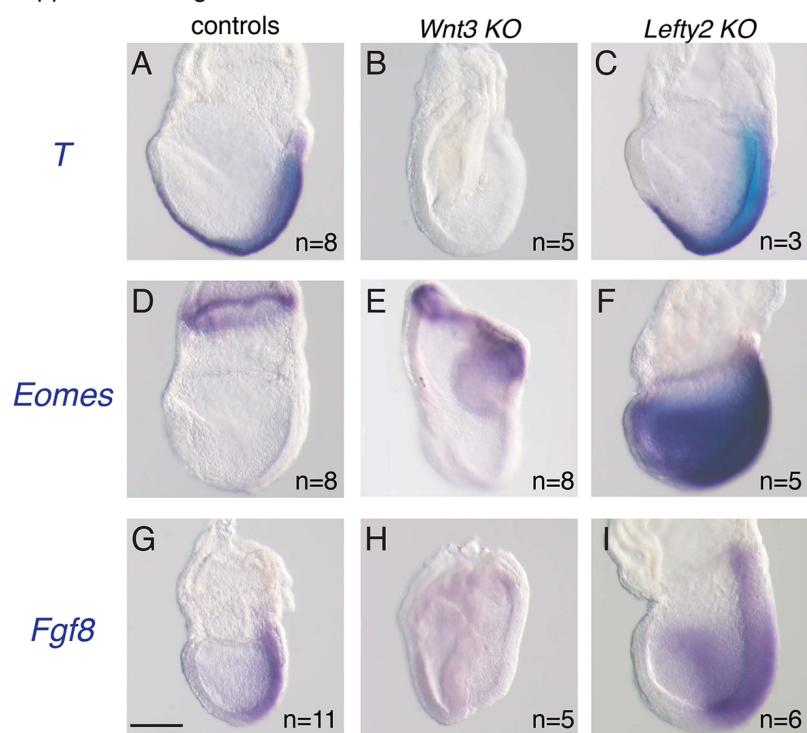


Fig. S6. Mesoderm marker expression in CRISPR/Cas9-mediated *Wnt3* and *Lefty2* KO embryos.

(A-I) Whole-mount in situ hybridization of control (A, D, G), CRISPR/Cas9-mediated *Wnt3* KO embryos (B, E, H), and CRISPR/Cas9-mediated *Lefty2* KO embryos (C, F, I) with *T* (A-C), *Eomes* (D-F), and *Fgf8* (G-I) probes.

Scale bar indicates 200 μ m.

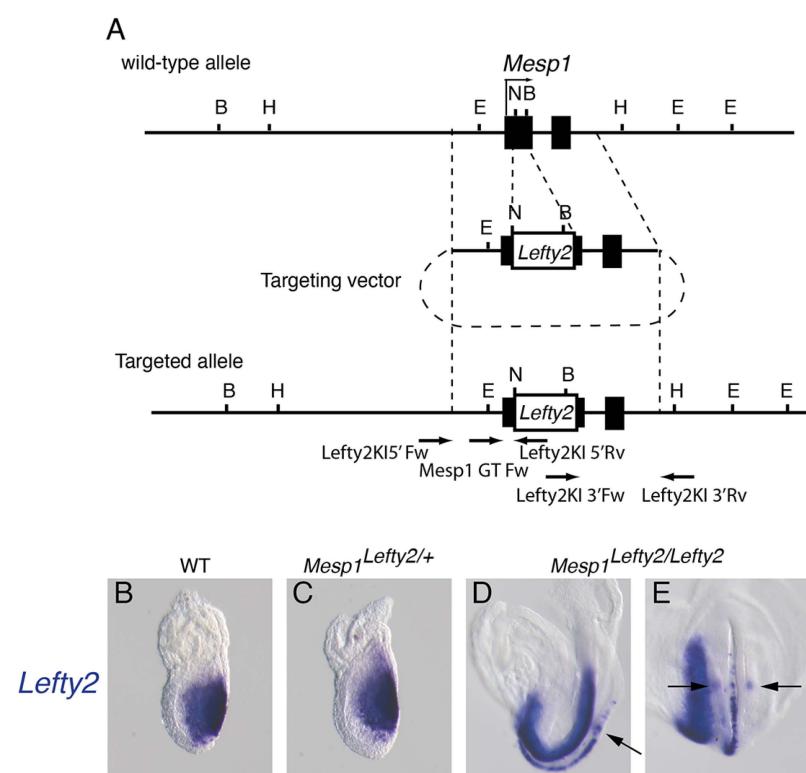


Fig. S7. Establishment of the *Mesp1Lefty2* mouse line.
 (A) Schematic representation of the targeting strategy for the *Mesp1Lefty2* mouse line.
 Restriction sites: B, BamHI; E, EcoRI; H, HindIII; N, NcoI.
 (B-D) Whole-mount *in situ* hybridization of *Lefty2* in wild-type (B) and *Mesp1Lefty2/+* (C) at E7.5, and *Mesp1Lefty2/Lefty2* (D, E) at E8.5. *Lefty2* expression in controls and *Mesp1Lefty2* embryos at E7.5 is indistinguishable because *Lefty2* and *Mesp1* expression largely overlaps.
 Arrows indicate ectopic *Lefty2* expression from the *Mesp1* locus, which was at the newly forming somite boundary.

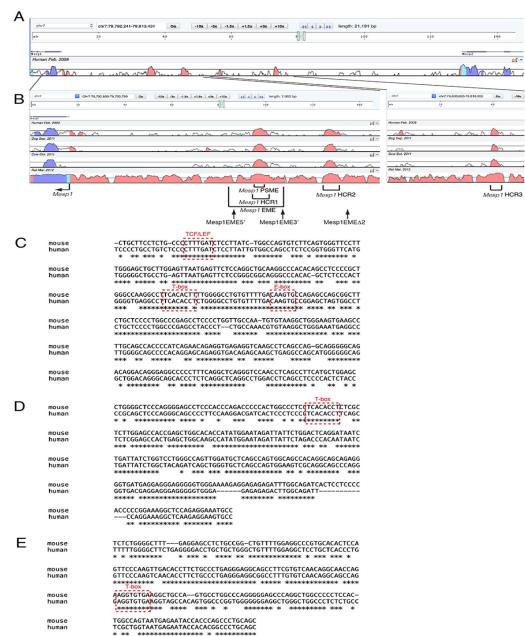
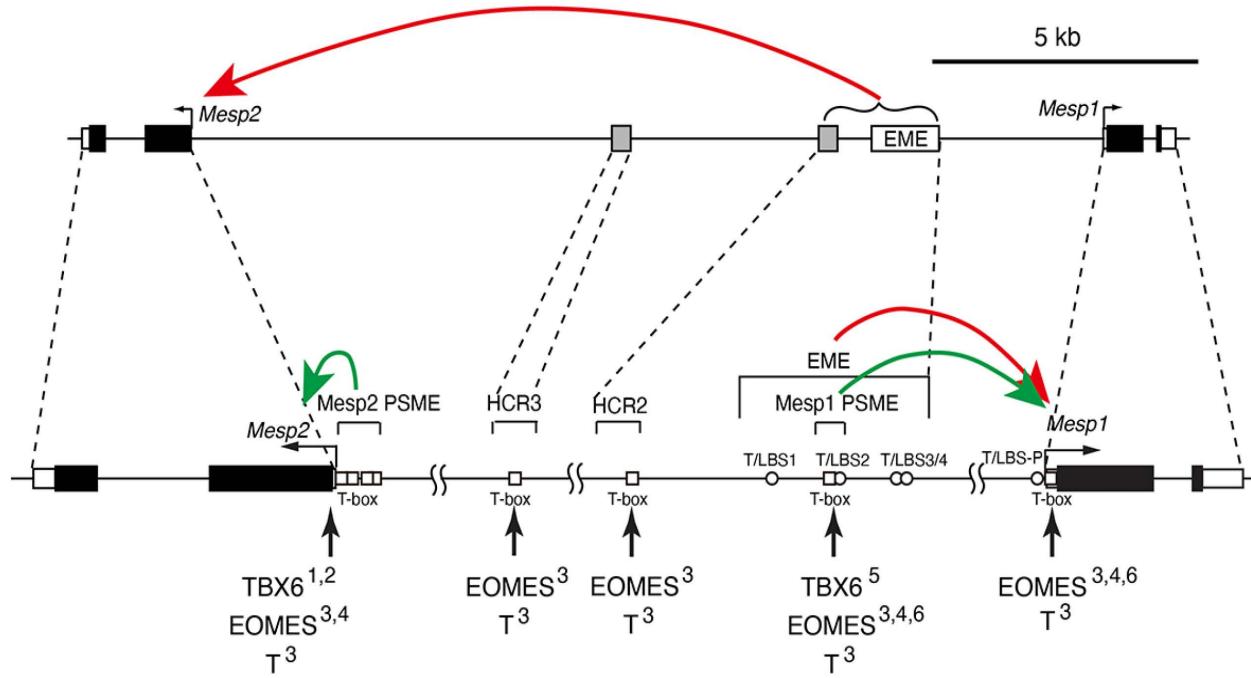


Fig. S8 Conservation of *Mesp1* enhancers among mammalian species.

(A) Sequence comparison using the VISTA tool. Mouse genomic DNA sequence of the *Mesp1* and *Mesp2* locus with that of human.
 (B) Sequence comparison using the VISTA tool. Mouse genomic DNA sequence of *Mesp1* upstream region with that of human, dog, cow, and rat. The *Mesp1* EME, *Mesp1* PSME position, and *Mesp1* highly conserved regions (HCR1,2, and 3) are labeled below. Arrows indicate the sgRNA positions in established EME deleted mouse lines.
 (C) Sequence comparison of HCR1 between mouse and human genomic DNA with labeled putative TCF/LEF binding sites, T-box, and E-box.
 (D,E) Sequence comparison of HCR2 and HCR3 between mouse and human genomic DNA with labeled putative T-box.

**Fig. S9. *Mesp1* and *Mesp2* expression is regulated via specific enhancers**

Schematic representation of *Mesp1* and *Mesp2* gene loci with enhancers and putative TCF/Lef-binding sites (T/LBS) and T box transcriptional factor-binding sites (T-box).

Red arrows indicate transcriptional regulation during the early mesoderm formation stage, and green arrows indicate transcriptional regulation during the somitogenesis stage.

The previously reported direct interactions of T-box transcriptional factors are listed below each T box binding site.

¹Yasuhiro et al. 2006, ²Yasuhiko et al. 2008, ³Totic et al. 2019, ⁴Costello et al. 2011, ⁵Sadahiro et al. 2018, ⁶van den Ameele et al. 2012

Table S1. Detailed genotyping analysis of F0 *Mesp1/Mesp2* dKO embryos

[Click here to download Table S1](#)

Table S2. F0 analysis of CRISPR/Cas9-mediated *Mesp1/2* single and double knock embryos

Dose of vectors	Injected	Two-cell	Obtained Embryos		Gene modified (homo)		
			Phenotype	Number	Mesp1	Mesp2	Large deletion
5 ng/μl pX330/Mesp1-1	54	42	Type I abnormal	8 2	8 (6) 2 (2)	n.e.	n.e.
5 ng/μl pX330/Mesp2-2	134	110	Type I Type II abnormal	2 17 1	n.e.	2 (2) 17 (17) 1 (0)	n.e.
5 ng/μl pX330/Mesp2-5	84	70	Type I Type II abnormal	16 9 1	n.e.	9 (0) 9 (4) 1 (0)	n.e.
5 ng/μl pX330/Mesp1-1 + 5 ng/μl pX330/Mesp2-2	103	81	Type I Type II Type III abnormal	4 12 6 4	4 (2) 11 (6) 6 (6) 4 (2)	4 (1) 12 (7) 6 (6) 4 (4)	0 2 3 0

(homo): Homozygously modified embryos. In these embryos, wild-type peaks were not observed by direct sequencing. The mutation did not necessarily cause a frame-shift. n.e. = not examined. The large deletion column is the number of embryos with a 17.6-kb deletion between *Mesp1* and *Mesp2* genes.

Table S3. List of differentially expressed genes (DEGs) of RNA-seq analysis using controls and *Mesp1*^{ΔNeo/ΔNeo} embryos

[Click here to download Table S3](#)

Table S4. Genotype of embryos or pups of the intercross of *Mesp1*^{EME} deletion mice

Crosses	Stage	WT	Hetero	Homo
<i>Mesp1</i> ^{EMEΔ1/+} intercross	E7.5	19	27	18
	pups	3	7	6
<i>Mesp1</i> ^{EMEΔ2/+} intercross	E7.5	12	31	11
	pups	14	25	10
<i>Mesp1</i> ^{EMEΔ1Δ2/+} intercross	E7.5	15	31	16
	pups	6	21	0

Table S5. Primer sets for ChIP assay and genotyping

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