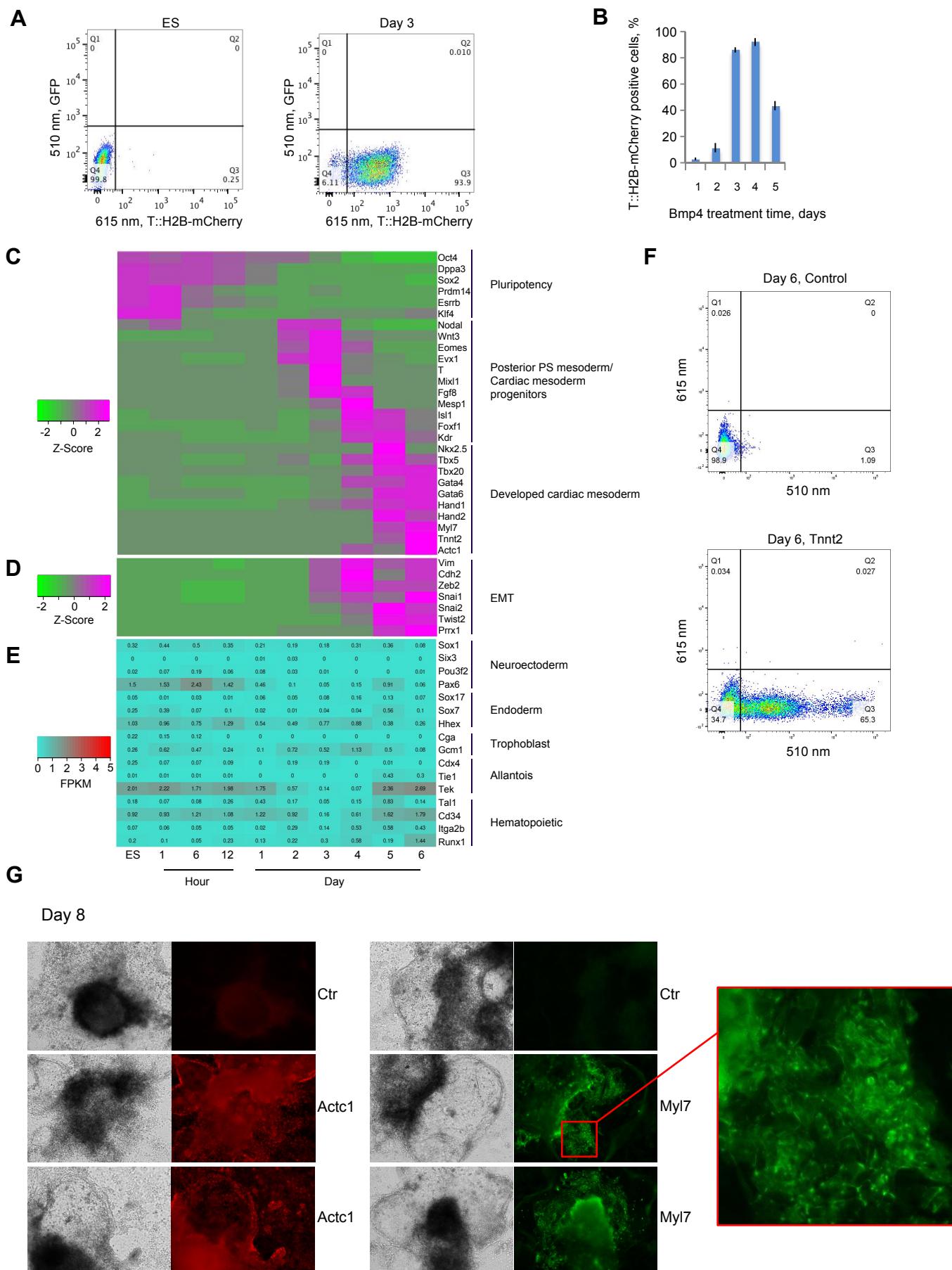


Figure S1

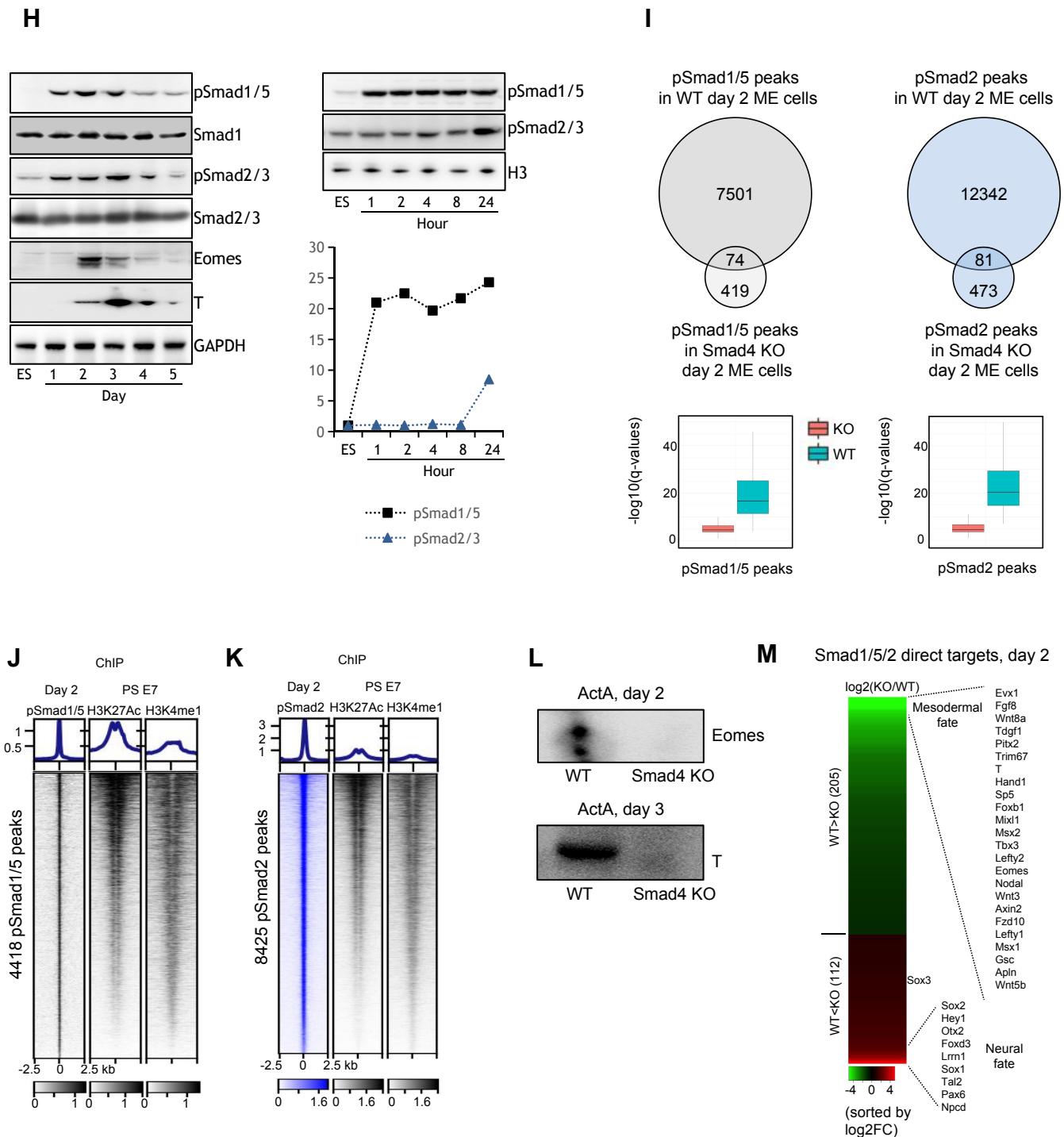


Fig. S1. Characterization of the *in vitro* differentiation system and DNA binding of Smads (related to Fig. 1)

- (A) Differentiation efficiency assessed by FACS using ES cell line with T::H2B-mCherry reporter inserted into the translation start site of T located on a BAC. FACS dot plots indicating that over 90% of cells express T at differentiation day 3.
- (B) Barplot showing fractions of cells expressing T::H2B-mCherry reporter during the differentiation time course assessed by FACS.
- (C-D) Heatmap representation of the relative expression (z-score) of pluripotency factors, mesoderm progenitor and cardiac mesoderm marker genes (D), and EMT marker genes (E) throughout the course of differentiation. (RNA-seq, average of two replicates).
- (E) Heatmap displaying the expression values (FPKM) of definitive endoderm and neuroectoderm marker genes throughout the course of differentiation. (RNA-seq, average of two replicates).
- (F) Cardiomyocyte formation efficiency assessed by FACS using day 6 cells expressing cardiomyocyte marker Tnnt2 (510nm).
- (G) Expression of cardiac markers Actc1 or Myl7 in differentiated WT aggregates at day 8 detected by immunofluorescence. Abbr.: Ctr, secondary antibody control.
- (H) Time-course of Smad1/5 or Smad2/3 phosphorylation and Eomes or T expression detected by Western blot analysis. Quantification of the Smad1/5 and Smad2/3 phosphorylation levels relative to ES.
- (I) Venn diagrams showing overlap of pSmad1/5 or pSmad2 peaks detected in WT and Smad4 KO day 2 ME cells. Peaks detected in Smad4 KO cells were excluded from further analyses. Boxplots showing distribution of the q-values for the corresponding peak sets.
- (J) Heatmaps centered on pSmad1/5 peaks outside promoters, displaying ChIP-seq reads of pSmad1/5 in day 2 ME cells and H3K27Ac and H3K4me1 in E7 posterior primitive streak [35].
- (K) Heatmaps centered on pSmad2 peaks outside promoters, displaying ChIP-seq reads of pSmad2 in day 2 ME cells and H3K27Ac and H3K4me1 in E7 posterior primitive streak [35].
- (L) Expression of Eomes or T in WT or Smad4 KO cells treated with Activin A (ActA) for 2 or 3 days, respectively.
- (M) Heatmap representation of the expression of pSmad1/5/2 direct target genes at day 2, sorted by log2 fold change (FC) of FPKM values in Smad4 KO or WT ME cells. The strongest activated and repressed target genes are listed on the right.

Figure S2

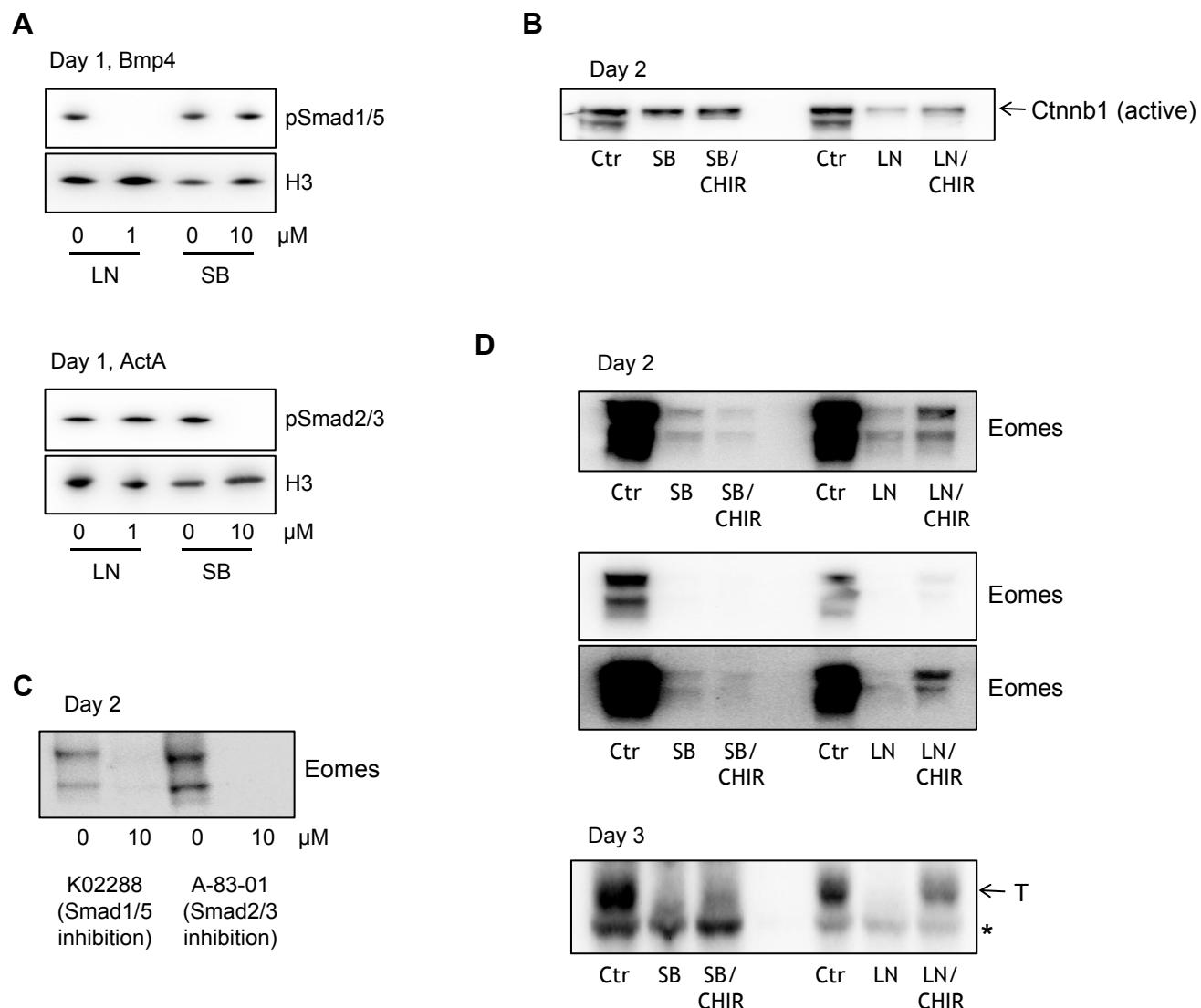


Fig. S2. Formation of proper nascent and committed cardiac mesoderm requires simultaneous activity of pSmad1/5, pSmad2/3, and WNT signaling (related to Fig. 2)

- (A)** 1 μ M LDN193189 (LN) or 10 μ M SB431542 (SB) selectively inhibit pSmad1/5 or pSmad2/3 phosphorylation, respectively. pSmad1/5 and pSmad2/3 are detected by Western blot analysis. pSmad1/5 and pSmad2/3 phosphorylation was induced by Bmp4 or ActA, respectively.
- (B)** Nuclear accumulation of active Ctnnb1 is selectively blocked by 1 μ M LDN (LN), as detected by Western blot analysis. Abbr.: Ctr, control.
- (C)** Eomes expression is repressed in day 2 ME cells by K02288 (inhibits Smad1/5 phosphorylation) as well as by A-83-01 (inhibits Smad2/3 phosphorylation).
- (D)** Western blot showing higher exposure of Fig. 2E, detecting Eomes expression (upper panel); Western blot analysis at low and high exposure showing Eomes protein expression in an independent experiment (middle panel). Independent experiment showing T expression by Western blot analysis (related to Fig. 2E; lower panel). Asterisk indicates a background band.

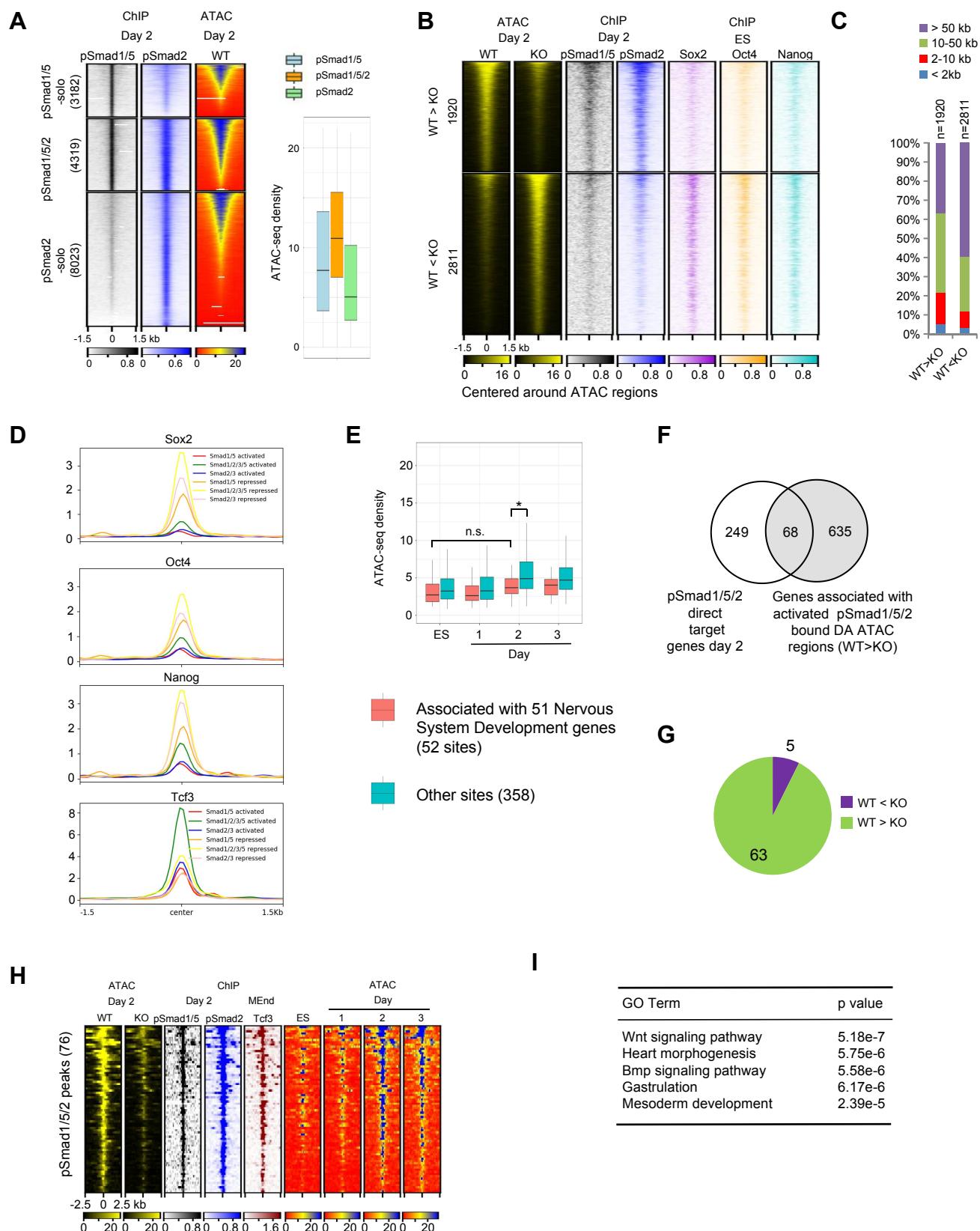
Figure S3

Fig. S3. Characterization of Smad-mediated chromatin regulation (related to Fig. 3)

- (A) Heatmaps, clustered by pSmad1/5 and pSmad2 occupancy and centered on the peak summits, displaying ChIP-seq reads of pSmad1/5 or pSmad2 and ATAC-seq reads from the same sites in day 2 ME cells. Boxplot of the quantification of normalized ATAC-seq density is shown on the right.
- (B) Clustered heatmaps displaying ATAC-seq reads in day 2 WT or Smad4 KO ME cells centered on sites activated or repressed by Smad activity, and the corresponding ChIP-seq reads of pSmad1/5, pSmad2, Sox2, Oct4 and Nanog.
- (C) Barplot showing quantification of distances between DA regions in Smad4 KO relative to WT cells and nearest gene promoters for groups defined in (B).
- (D) Average ChIP-seq density profiles of indicated TFs in clusters defined in Fig. 3C.
- (E) Boxplot of the quantification of normalized ATAC-seq density during the differentiation course in sites associated with Nervous system Development GO term and other p-Smad2-solo bound sites defined in (Fig. 3C, Fig. 3G). (Student's t-test, * $p < 5e-2$, n.s., not significant).
- (F) Venn diagram showing overlap of pSmad1/5/2 direct target genes with genes bound by activated pSmad1/5/2-containing DA regions.
- (G) Pie chart showing that the majority of pSmad1/5/2 direct targets associated with activated pSmad1/5/2-bound DA regions (see Fig. S3E) are Smad-activated (WT > Smad4 KO).
- (H) Heatmaps centered on 76 pSmad1/5/2 peaks associated with 63 activated pSmad1/5/2 targets from (F), displaying ATAC-seq reads in WT or Smad4 KO day 2 ME cells, ChIP-seq reads of indicated TFs, and ATAC-seq reads during differentiation.
- (I) GO terms enriched among the 63 activated pSmad1/5/2 target genes defined in (F).

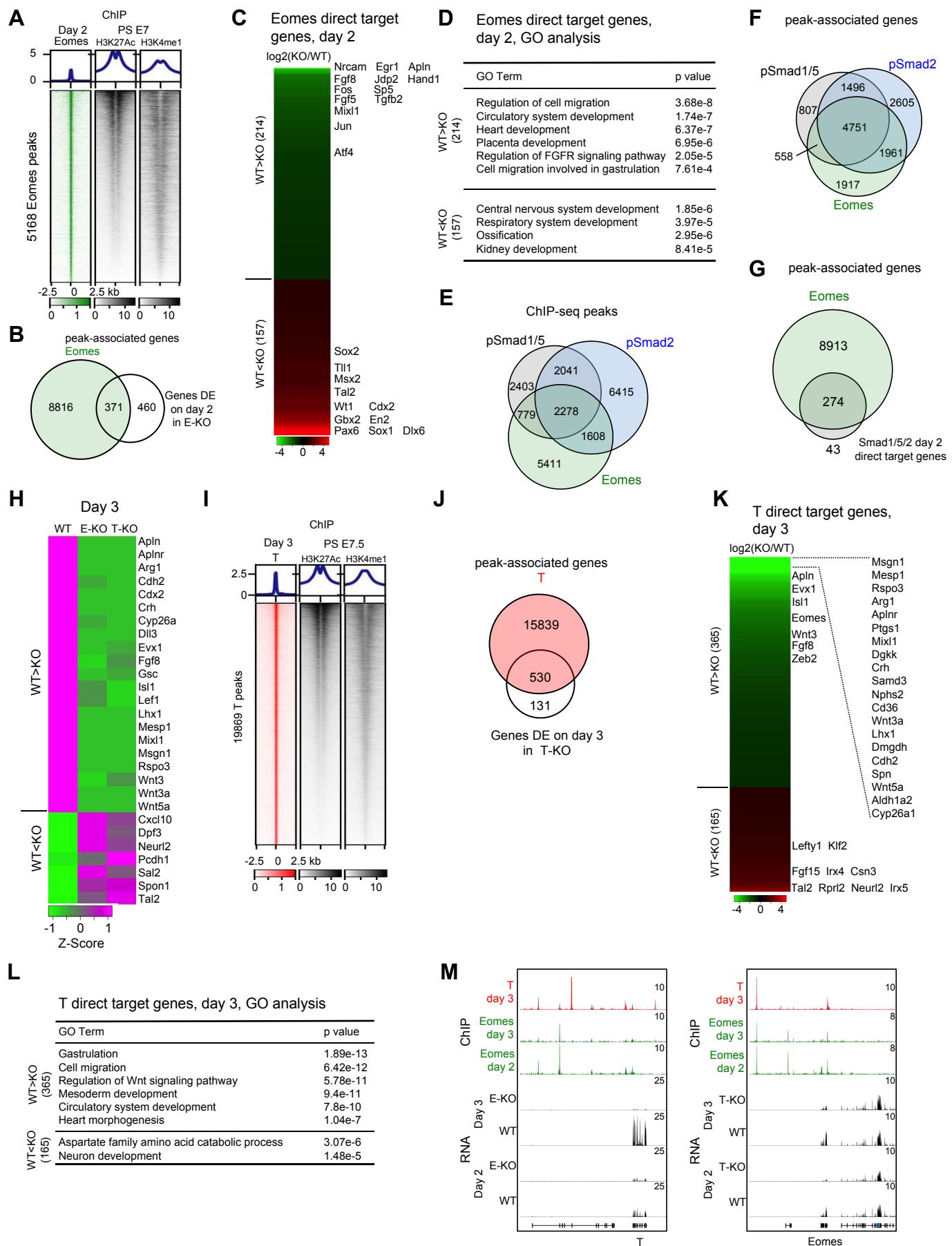
Figure S4

Fig. S4. DNA binding and target genes of Eomes and T (related to Fig. 5)

- (A) Heatmaps centered on promoter-far Eomes peaks, displaying ChIP-seq reads of Eomes in day 2 ME cells, and H3K27Ac and H3K4me1 in E7 posterior primitive streak [35].
- (B) Venn diagram showing overlap between genes bound by Eomes and genes DE between WT and Eomes KO cells. Genes in the intersection are putative direct targets of Eomes.
- (C) Heatmap representation of Eomes day 2 direct target gene expression, sorted by log₂ fold change (FC) of FPKM values in Eomes KO or WT ME cells. Selected activated and repressed target genes are listed on the right.
- (D) GO terms enriched among activated (WT>KO) or repressed (WT<KO) Eomes direct target genes.
- (E) Venn diagram showing overlap between pSmad1/5, pSmad2 and Eomes DNA binding sites in day 2 ME cells.
- (F) Venn diagram showing overlap between genes bound by pSmad1/5, pSmad2, and Eomes in day 2 ME cells.
- (G) Venn diagram showing overlap between genes bound by Eomes and direct target genes of pSmad1/5/2 in day 2 ME cells.
- (H) Heatmap representation of the expression of selected Eomes and T direct target genes in WT, Eomes KO (E-KO) or T KO (T-KO) day 3 ME cells. (RNA-seq, average of two replicates)
- (I) Heatmaps centered on promoter-far T peaks, displaying ChIP-seq reads of T in day 3 ME cells, and H3K27Ac and H3K4me1 in E7.5 posterior primitive streak [35].
- (J) Venn diagram showing overlap between genes bound by T and genes DE between WT and T-KO cells. Genes in the intersection are putative direct targets of T.
- (K) Heatmap representation of the expression of T direct target genes at day 3, sorted by log₂ fold change (FC) of FPKM values in T-KO relative to WT ME cells. Selected activated or repressed target genes are listed on the right.
- (L) GO terms enriched among activated (WT>KO) or repressed (WT<KO) day 3 direct target genes of T.
- (M) T is a direct target of Eomes, and vice versa. Snapshots of ChIP-seq and RNA-seq tracks in WT, E-KO or T-KO ME cells at T and Eomes loci.

Figure S5

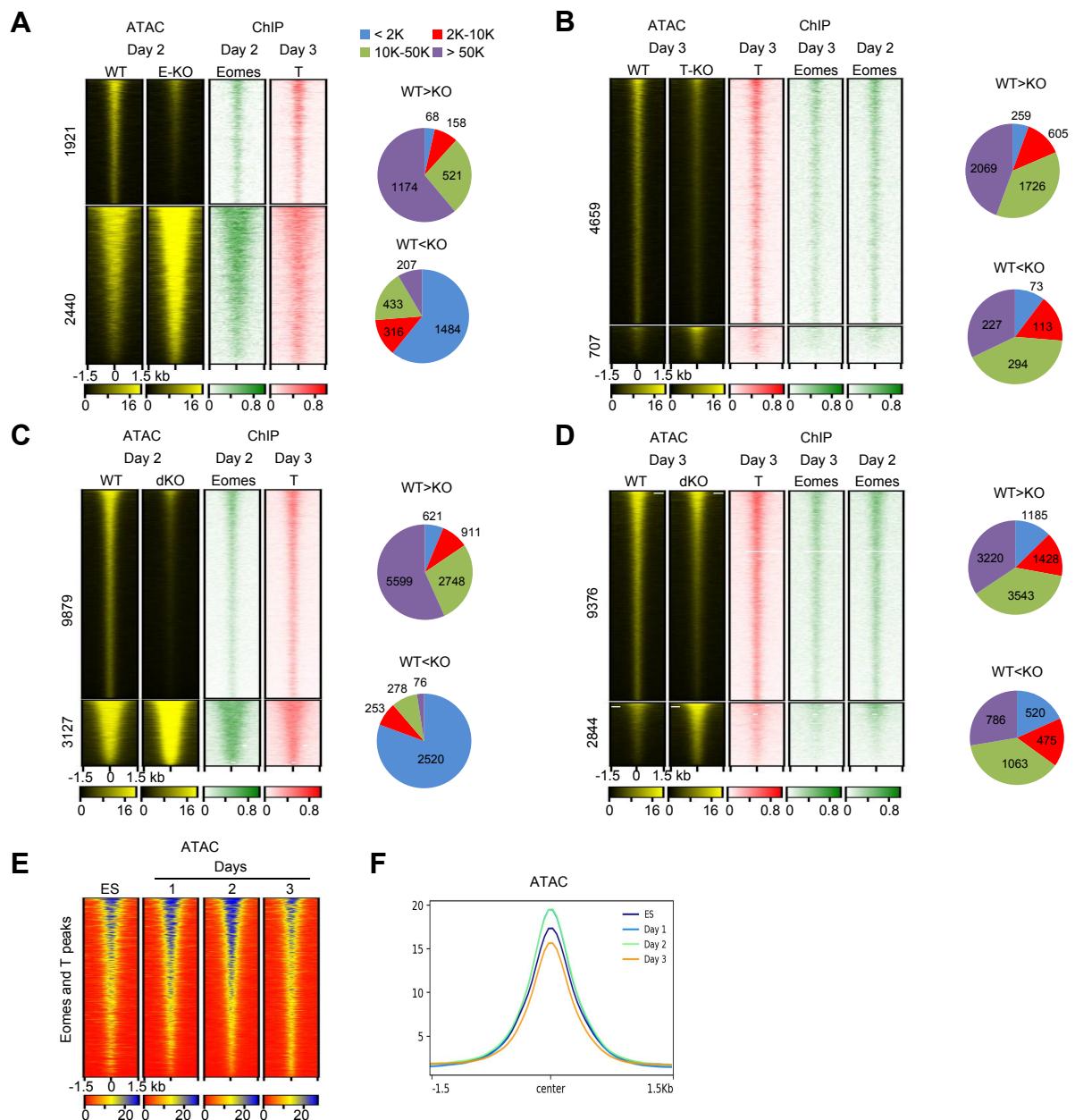


Fig. S5. Characterization of Eomes- and/or T-mediated chromatin regulation (related to Fig. 6)

- (A-D) Clustered heatmaps displaying ATAC-seq reads in WT or (A) E-KO, (B) T-KO, (C,D) double KO (dKO) ME cells, centered on activated or repressed DA sites, and the corresponding ChIP-seq reads of indicated TFs (left). Pie charts showing quantification of distances between the corresponding activated or repressed DA regions and nearest gene promoters (right).
- (E) Heatmap displaying ATAC-seq reads centered on activated DA regions co-occupied by Eomes and T (see Fig. 6D).
- (F) Average ATAC-seq density profiles in clusters from (E).

Table S1. GO term analysis for genes bound by pSmad1/5 and pSmad2 (related to Fig. 1).

GO Term	pSmad1/5 and pSmad2 common targets (6247)	pSmad1/5 only targets (1365)	pSmad2 only targets (4566)
Nervous system development	1.61e-27	2.84e-5	1.2e-5
Heart development	1.13e-18	ND	ND
Wnt signalling	4.4e-11	ND	ND
Mesoderm development	1.95e-8	ND	ND
Gastrulation	3.8e-8	ND	ND
TGFbeta signalling pathway	3.7e-7	ND	ND
Regulation of EMT	3.31e-5	ND	ND

Table S2. GO term analysis for direct target genes of pSmad1/5/2 (related to Fig. 1).**A** pSmad1/5/2 direct targets, day 1

	GO Term	p value
WT>KO (110)	Mesoderm formation	1.91e-9
	BMP signaling pathway	1.63e-5
	Wnt signaling pathway	2.29e-5
WT<KO (89)	Nervous system development	4.21e-14
	Neuron differentiation	3.98e-9

B pSmad1/5/2 direct targets, day 2

	GO Term	p value
WT>KO (205)	Wnt signaling pathway	2.68e-11
	Heart development	3.17e-10
	Vasculature development	4.03e-8
	BMP signaling pathway	1.93e-7
	EMT	8.99e-7
	Mesoderm formation	4.87e-6
WT<KO (112)	Nervous system development	1.2e-11
	Neuron fate commitment	4.56e-5

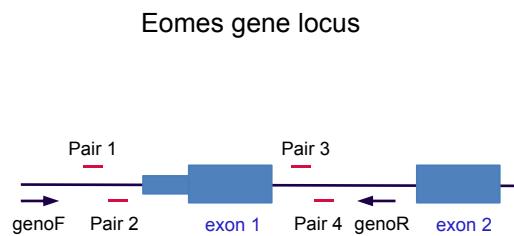
Table S3. Lists of pSmad1/5, pSmad2, Eomes, and T direct target genes and genes associated with indicated sets of DNA loci (related to Figs. 1 - 6).

Click here to download Table S3

Table S4. Oligonucleotides used for generation and genotyping of knock-out cells. Primers used for RT-PCR.

Single stranded oligonucleotides for Eomes KO

Pair 1:	CACCGACATGGTCTACCCACGGCAT AAACATGCCGTGGGTAGACCATGTC	F R
Pair 2:	CACCGTCCGGCGCCATAGAGCCACC AAACGGTGGCTCTATGGCGCCGGAC	F R
Pair 3:	CACCGTACGTAGATCTGCCCTCAA AAACTTGAGGGCAGATCTACGTGAC	F R
Pair 4:	CACCGGTTGCAGCAGGGGATTG AAACCAAATGCCCTGCTGCAAACC	F R

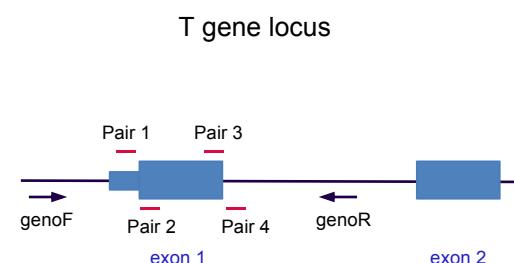


Eomes KO genotyping primers

genoF: TGAAGGCCAGGAAGTCAGAAG
genoR: GTCACCAGCTAAACCGACTTC

Single stranded oligonucleotides for T KO

Pair 1:	CACCGCGCCACCCCTCTCCACCTTCC AAACGGAAAGGTGGAGAGGGTGGCGC	F R
Pair 2:	CACCGTCGCCGGGCACAGAGAGCGC AAACCGCGCTCTGTGCCCGGCGAC	F R
Pair 3:	CACCGACGCACCTACCTGCCGTTCT AAACAGAACGGCAGGTAGGTGCGTC	F R
Pair 4:	CACCGGCGAGTGCCCCTCGCCTCG AAACCGAGGCAGGGGACTCGCC	F R



T KO genotyping primers

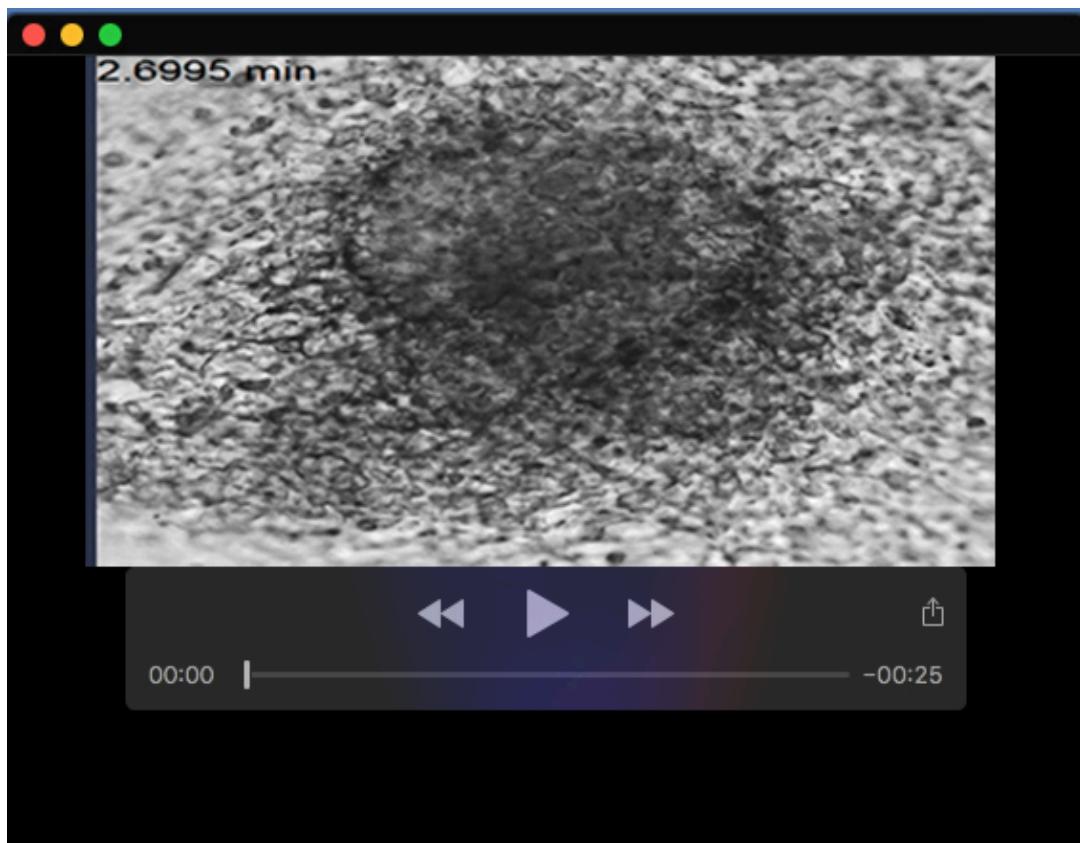
genoF: GCAGCTGCTCGGTACTTCAAAG
genoR: CAGCAGAGGGACAAATCTAGCCAAG

Primers used for qRT-PCR assays

CTTCTAATGGAGCCCCACCTG	F	Wnt3
GGGCCAGGGACCACCAAAT	R	
CTGGCGTACATGTTGAGCCT	F	Nodal
GGTCACGTCCACATCTGCG	R	
GGGACAACATCGATTATCCA	F	Eomes
GGGCTTGAGGCAGGAAAGTGTG	R	
GGTATTCCCAATGGGGGTGG	F	T
TGTGTGGAGGGGAGAGAGAG	R	
GGATCCCCGCCTGCCTA	F	Mesp1
GCTGCTGAAGAGCGGAGAT	R	
CCCCGTCCATCAGAGAGCTA	F	Actc1
TGGGTTCTGTAGGCCTGCTA	R	
ACTGGGAAATGATGAGAATGGC	F	Pmm2
CCCCCAGATGCCCTTGAATA	R	

Table S5. RNA-seq data (FPKM values) of the RefSeq genes for the entire differentiation course and indication of genes associated with pSmad1/5, pSmad2, Eomes, and T ChIP-seq peaks; all direct target genes of pSmad1/5, pSmad2, Eomes, and T; and all genes associated with sites opened or closed in Smad4, Eomes and/or T KO cells compared to WT cells.

[Click here to download Table S5](#)



Movie 1. Capture of contracting cells on differentiation day 8.