Supplementary Figure Legends

Supplementary Figure S1. Genome-wide identification and characterization of Smad1/5-binding sites in HUVECs

(A) Dose response study of BR-Smad phosphorylation after BMP-6 or BMP-9 stimulation in HUVECs. HUVECs were starved overnight and stimulated with the indicated concentration of BMP-6 or BMP-9 for 1 h. The cells were then subjected to immunoblot analysis to determine the phospho-Smad1/5 (pSmad1/5) protein level. α-tubulin was used as a loading control.

(B) Comparison of induction of *ID1* after BMP-6 or BMP-9 stimulation. HUVECs were starved overnight and stimulated with BMP-9 (1 ng/ml) or BMP-6 (50 ng/ml) for indicated time periods. The cells were then subjected to quantitative RT-PCR analysis for *ID1*. Values were normalized to the amount of housekeeping *GAPDH* mRNA. The data are the mean of triplicate values ± standard deviation.

(C) Comparison of protein level of phospho-Smad1/5/8 after BMP-6 or BMP-9 stimulation. HUVECs were starved overnight and stimulated with BMP-9 (1 ng/ml) or BMP-6 (50 ng/ml) for 1 h. The cells were then fractionated into nuclear and cytosolic fractions and subjected to immunoblot analysis to determine the phospho-Smad1/5/8 (pSmad1/5/8) protein level. Separation of the fractions was verified by immunoblotting of histone deacetylase 1 (HDAC-1) (nuclear marker) and α-tubulin (cytosolic marker), which are also served as a loading control. TCL indicates total cell lysates.

(D) Dose response study of BR-Smads phosphorylation after BMP-4 stimulation in PASMCs. PASMCs were starved overnight and stimulated with the indicated concentration of BMP-4 for 1 h. The cells were then subjected to immunoblot analysis to

determine the phospho-Smad1/5/8 (pSmad1/5/8) protein level. α-tubulin was used as a loading control.

(E) Conventional RT-PCR analysis of BR-Smad genes. RNA samples from HUVECs, PASMCs, HepG2, and HMEC-1 cells were analyzed by conventional RT-PCR for expression of BR-Smad genes. Primers used for SMAD1, SMAD5, SMAD8, and SMAD4 specifically detect the endogenous transcripts. GAPDH was used as a loading control. H_2O indicates no template control reactions, where the cDNA was substituted with nuclease-free water.

(F) Validation of anti-Smad1/5 antibody used for chromatin immunoprecipitation (ChIP) procedure. HEK293T cells were transfected with FLAG tagged-Smad constructs and subjected to immunoblot analysis with anti-Smad1/5 antibody or anti-FLAG (M2) antibody.

(G) Immunoprecipitation (IP) was performed with the same procedure to chromatin immunoprecipitation (ChIP) experiments. Briefly, $1.5x10^6$ cells were starved overnight and stimulated with BMP-9 (1 ng/ml) for 1.5 h, and then cross-linked with 1% formaldehyde. The cross-linked cells were harvested by scraping, pelleted, resuspended in 1 ml of lysis buffer, and sonicated. Protein-DNA complex were immunoprecipitated for 8 h by anti-mouse IgG Dynabeads preincubated with 5 μg of anti-Smad1/5 antibody. The beads were then washed five times with RIPA buffer and once with TE buffer. Proteins were eluted using SDS sample buffer and subjected to immunoblot analysis with anti-Smad1/5/8 antibody.

(H) Time course study of Smad1/5 binding to *ID1*, *HEY1* locus or *HPRT1* 1^{st} intron. HUVECs were starved overnight and stimulated with BMP-9 (1 ng/ml) or BMP-6 (50

ng/ml) for indicated time periods and subjected to ChIP assays with anti-Smad1/5 antibody. The ChIP samples were quantified by real-time PCR with locus specific primers (see Table S4) and normalized to input DNA. The dashed line indicates 0.01% of input. The data are the mean of triplicate values ± standard deviation.

(I) Visualization of several Smad1/5 binding regions. Genomic loci are shown together with the results of ChIP-seq. Red peaks represent ChIP regions (top panel). The direction of transcription is shown by the arrow beginning at the transcription start site (TSS). Horizontal blue bars represent the positions of PCR primers used in this study.

(J) Conservation plots of Smad1/5 binding regions. The mean phastCons conservation score was calculated by CisGenome and was plotted against the distance from peak summits (within 1.5 kb from peak summit).

Supplementary Figure S2. *De novo* motif search for overrepresented motifs in Smad1/5 binding regions in different cell types.

(A-C) Results of *de novo* motif discovery performed on 815 common Smad1/5 binding sites shared with HUVECs and PASMCs (A), 2,935 HUVEC-specific binding sites (B), and 1,908 PASMC-specific binding sites (C). For each predicted motif, matched motifs in the database are indicated with TOMTOM *p*-value.

Supplementary Figure S3. Genome-wide analysis of the relation between Smad1/5 binding and gene expression profiles.

(A) Graphical representation of microarray expression data of ID proteins after BMP-6 or BMP-9 stimulation.

(B) Graphical presentation of the number of up- or down-regulated genes associated with Smad1/5 binding regions. The number of the genes up- or down-regulated more than 2 fold relative to time 0 are graphically displayed (red and blue, respectively). The genes associated with Smad1/5 binding regions are also represented (light red or light blue, respectively).

(C) A screenshot of chromosome 2 with DNA-binding profiles of Smad1/5 treated with BMP-9 and histone modification markers. RefSeq Genes track (Green) shows known protein-coding genes taken from the NCBI mRNA reference sequences collection (RefSeq). The red peaks represent Smad1/5 binding, and blue represent histone modification markers. The data for H3K4me1 and H3K27ac are derived from ENCODE projects. Each vertical black line above the peak represents the regions with signal intensity exceeded the threshold value. Areas where Smad1/5 binding regions are overlapped with the H3K4me1-positive or the H3K27ac-positive regions are also indicated as black vertical lines (‘overlap’ track).

Supplementary Figure S4. *De novo* prediction of Smad1/5 binding motif.

(A) The sequences of 170 Smad1/5 binding regions associated with putative direct target genes were analyzed for overrepresented motifs using MEME. The top 5 motifs selected by MEME software are named as MEME1 to MEME5 and displayed as sequence logos. The sequence logo of MEME2 is shown in Figure 4A.

(B) TFBS enrichment calculated against chromosome-matched control sequences. Enrichment of transcription factor binding site (TFBS) in Smad1/5 binding regions was calculated. For obtaining background data, chromosome-matched sequences were

generated randomly for 1,000 times. In order to calculate the frequency of TFBS-positive sequences, MATCH score of position-specific scoring matrix (PSSM) for each transcription factor was computed. The highest MATCH score (HMS) was assigned to each sequence, and the number of sequences with HMS greater than or equal to a threshold was counted. Data are given as boxplot. The circles represent outlier values. The black circles indicate the number of matched motifs observed in the Smad1/5 binding regions.

(C) MEME2 motif occurs in about 45% of all Smad1/5 binding regions in HUVECs treated with BMP-6 and PASMCs treated with BMP-4.

(D) Fraction of peaks with at least one MEME2 motif. ChIP-seq peaks of Smad1/5 in HUVECs treated with BMP-9 are ranked by peak height. Fraction of peaks with at least one MEME2 motif is calculated for every 100 peak and plotted. Peaks with motifs are enriched in the high ranked peaks.

(E) Distribution of MEME2 motif around the peak summits. (Upper) Schematic presentation of the analysis. The MEME2 motif was mapped in regions within 500 bp from the peak summits using CisGenome with the likelihood ratio more than 500 (default value). Data from all peaks were accumulated and presented as a line histogram. (Lower) The number of the MEME2 motif around the peak summits was counted and plotted in a 7-bp sliding window against the distance from the summits (within 500 bp from the summits) (blue). The motifs closest to the summits are located within 100 bp from the peak summit (1^{st} motif; green). Five separate matched control regions were randomly chosen by CisGenome and used as a control. The number of the MEME2 motif in those regions was counted and the average was plotted (red).

(F) Distribution of identified motifs around the peak summits. The number of each identified motif is counted and plotted in a 7-bp sliding window against the distance from peak summits (within 500 bp from peak summits), as in Figure 4D.

Supplementary Figure S5. Validation of GGAGCC sequence as a novel BMP responsive element.

(A) Schematic presentation of the enhancer sequences and luciferase reporter constructs. Smad1/5 binding regions, which are located in *BMPR2* intron 3 and the *JAG1* promoter, contain GGAGCC. These regions were subcloned into pGL4 vector with adenoviral major late promoter (MLP). Reporter constructs named SBEmut contain mutation in SBE.

(B) pGL4-JAG1 reporter constructs were introduced into HUVECs using lentiviral vector system in order to evaluate their enhancer activity. The cells were stimulated with indicated doses of BMP-9 or BMP-6 and then they were harvested and assayed for luciferase activity at 12 h after stimulation. The data are the mean of triplicate values ± standard deviation.

(C) PASMCs were transfected with indicated reporter constructs to evaluate their enhancer activity. The cells were stimulated with BMP-4 (30 ng/ml) at 24 h after transfection, and then they were harvested and assayed for luciferase activity at 16 h after stimulation. The data are the mean of triplicate values ± standard deviation.

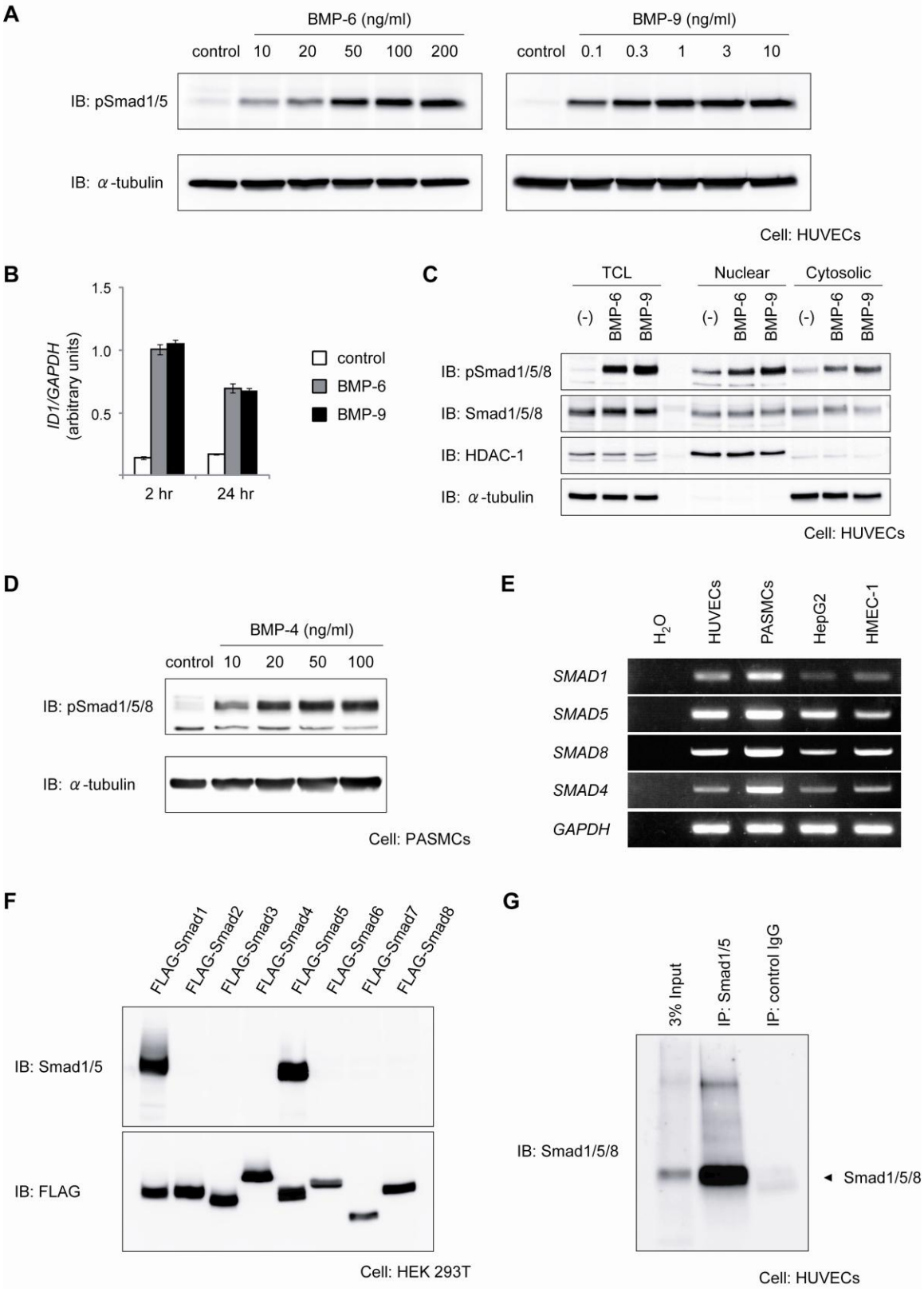
(D and E) HMEC-1 cells (D) and HepG2 cells (E) were transfected with indicated reporter constructs to evaluate their enhancer activity. The cells were stimulated with BMP-9 (1 ng/ml) for HMEC-1 or BMP-6 (50 ng/ml) for HepG2 at 24 h after transfection,

and then they were harvested and assayed for luciferase activity at 16 h after stimulation. The data are the mean of triplicate values ± standard deviation.

Supplementary Figure S6. The Notch ligand JAG1 is a direct target gene of Smad1/5.

(A) HUVECs were starved overnight and stimulated with 1 ng/ml BMP-9 for 2 h. To access whether activation of *JAG1* by BMP-9 was independent of *de novo* protein synthesis, cells were treated with cycloheximide (CHX, 10 μM) for 30 min before the treatment of BMP-9. Cells were then subjected to quantitative RT-PCR analysis for *JAG1*. Values were normalized to the amount of housekeeping *GAPDH* mRNA. The data are the mean of triplicate values ± standard deviation.

(B) HUVECs were transfected with siRNA against Smad4 or no template control (NTC). Next day, cells were starved overnight, stimulated with 1 ng/ml BMP-9 for 2 h and subjected to quantitative RT-PCR analysis for *JAG1*. Values were normalized to the amount of housekeeping *GAPDH* mRNA. The data are the mean of triplicate values ± standard deviation.


A
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control 10 20 50 100 200
BMP-9 (ng/ml)
control 0.1 0.3 1 3 10
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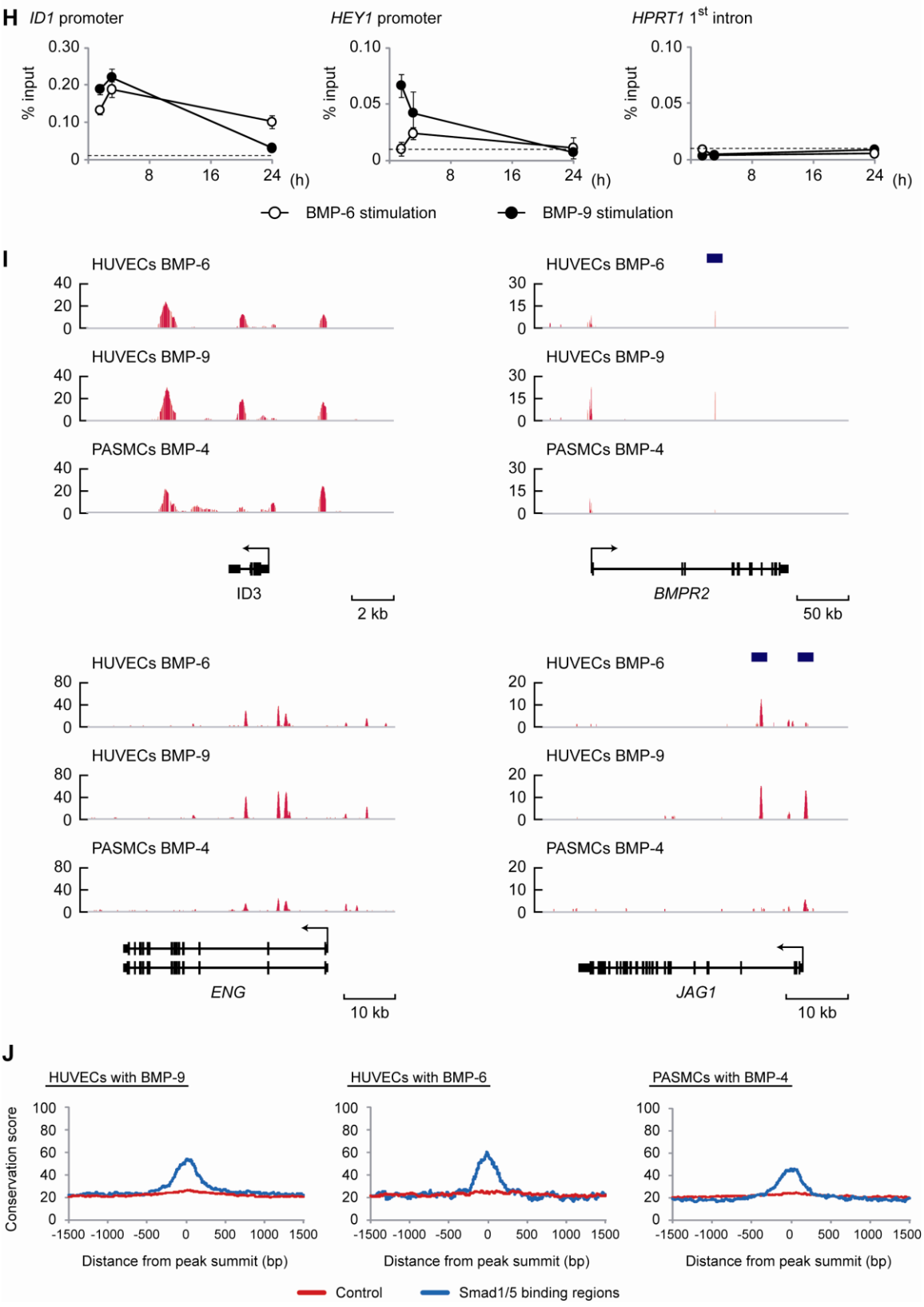
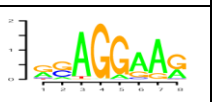
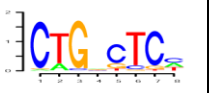
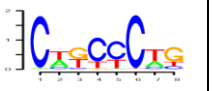
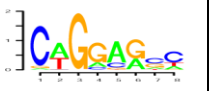
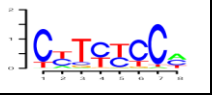
IB: α-tubulin
Cell: HUVECs
B
ID1/GAPDH (arbitrary units)
1.5
1.0
0.5
control
BMP-6
BMP-9
2 hr
24 hr
C
TCL
Nuclear
Cytosolic
(-) BMP-6 BMP-9
IB: pSmad1/5/8
IB: Smad1/5/8
IB: HDAC-1
IB: α-tubulin
Cell: HUVECs
D
BMP-4 (ng/ml)
control 10 20 50 100
IB: pSmad1/5/8
IB: α-tubulin
Cell: PASMCs
E
H2O
HUVECs
PASMCs
HepG2
HMEC-1
SMAD1
SMAD5
SMAD8
SMAD4
GAPDH
F
FLAG-Smad1
FLAG-Smad2
FLAG-Smad3
FLAG-Smad4
FLAG-Smad5
FLAG-Smad6
FLAG-Smad7
FLAG-Smad8
IB: Smad1/5
IB: FLAG
Cell: HEK 293T
G
3% Input
IP: Smad1/5
IP: control IgG
IB: Smad1/5/8
◄ Smad1/5/8
Cell: HUVECs

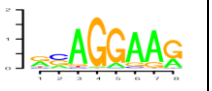
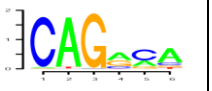
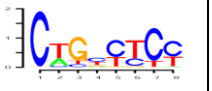
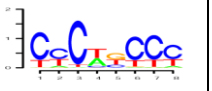
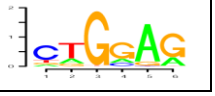
Supplementary Figure S1 (*Continued*)



A. Common Smad1/5 binding sites shared with HUVECs and PASMCs (n=815)

	motif	*p*-value	
motif1	M00339 (c-Ets-1)	1.3E-06	
	M00340 (c-Ets-2)	1.4E-06	
	M00971 (Ets)	2.9E-06	
motif2	M00005 (AP-4)	2.8E-04	
	M00983 (MAF)	5.4E-04	
	M00199 (AP-1)	5.4E-04	
motif3	M00629 (Eve)	4.5E-05	
motif4	M01207 (ETS2)	9.1E-04	
	M00743 (c-Ets-1)	1.6E-03	
	M00321 (Muscle)	2.5E-03	
motif5	M01295 (ATF5)	3.3E-04	
	M00649 (MAZ)	1.1E-03	
	MA0079.2 (SP1)	1.1E-03	

B. HUVEC-specific Smad1/5 binding regions (n=2,935)

	motif	*p*-value	
motif1	M00340 (c-Ets-2)	3.0E-07	
	M00339 (c-Ets-1)	3.5E-07	
	M01207 (ETS2)	5.3E-07	
motif2	M00804 (E2A)	1.2E-04	
	M00929 (MyoD)	7.9E-04	
	M00773 (MYB)	1.9E-03	
motif3	M00701 (SMAD3)	2.5E-04	
	M00974 (SMAD)	4.3E-04	
	M00733 (SMAD4)	2.3E-03	
motif4	MA0079.2 (SP1)	5.1E-05	
	M00720 (CAC-binding)	5.5E-05	
	M00008 (Sp1)	7.3E-05	
motif5	M00808 (Pax)	2.8E-03	
	M00238 (Barbie)	4.0E-03	

C. PASMC-specific Smad1/5 binding regions (n=1,908)

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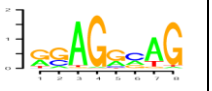
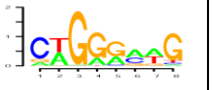
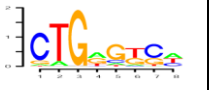
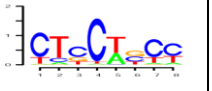
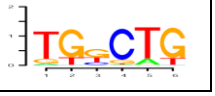
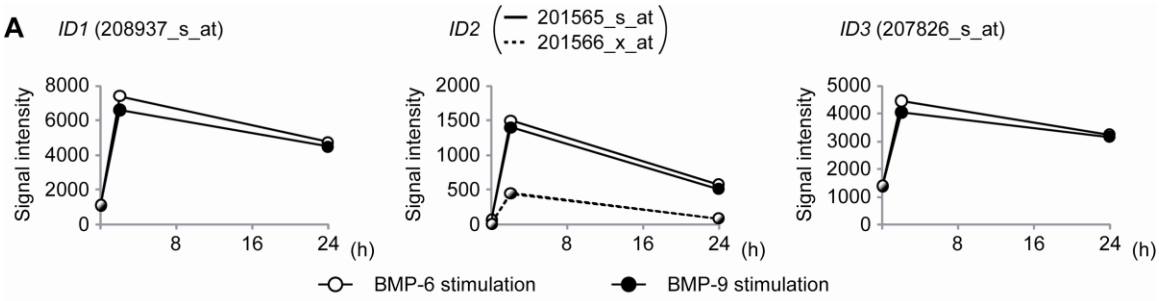
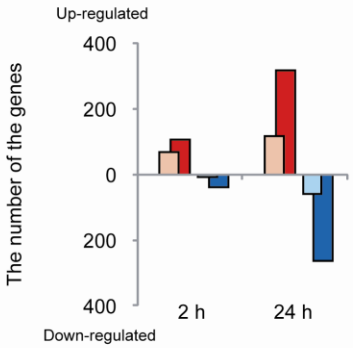
motif1	M00706 (TFII-I)	4.1E-04	
	M00658 (PU.1)	5.0E-04	
	M00971 (Ets)	5.8E-04	
motif2	M00629 (Eve)	5.2E-04	
	M00704 (TEF-1)	8.8E-04	
motif3	M00983 (MAF)	8.1E-05	
	M00037 (NF-E2)	1.5E-04	
	M00005 (AP-4)	2.1E-04	
motif4	M01119 (KAISO)	2.0E-03	
	M00238 (Barbie)	3.1E-03	
	M01288 (NeuroD)	3.2E-03	
motif5	M00733 (SMAD4)	1.2E-04	
	M00687 (alpha-CP1)	2.1E-04	
	M00701 (SMAD3)	4.4E-04	



B



Genes expressed in HUVECs
n = 8,544

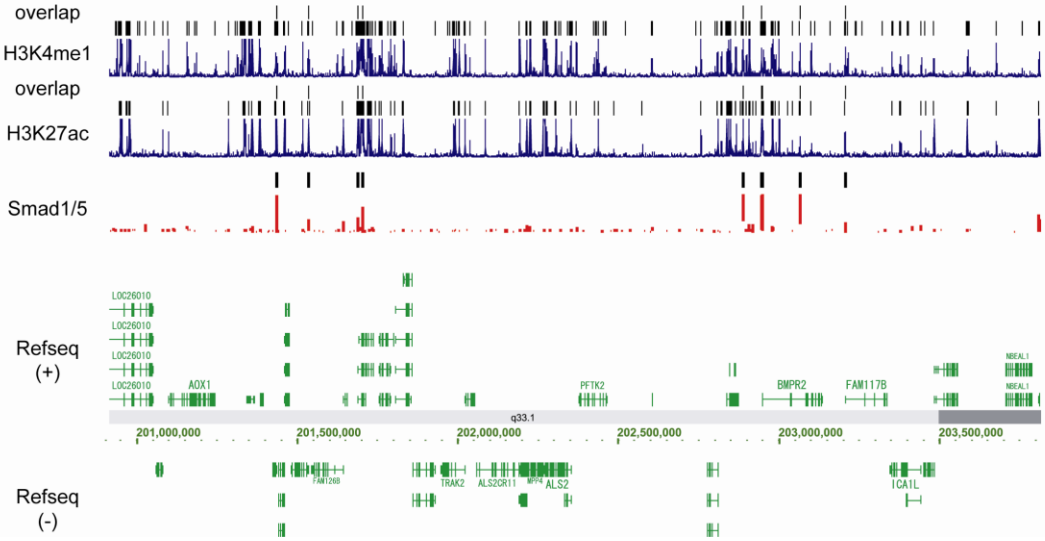
Up-regulated genes

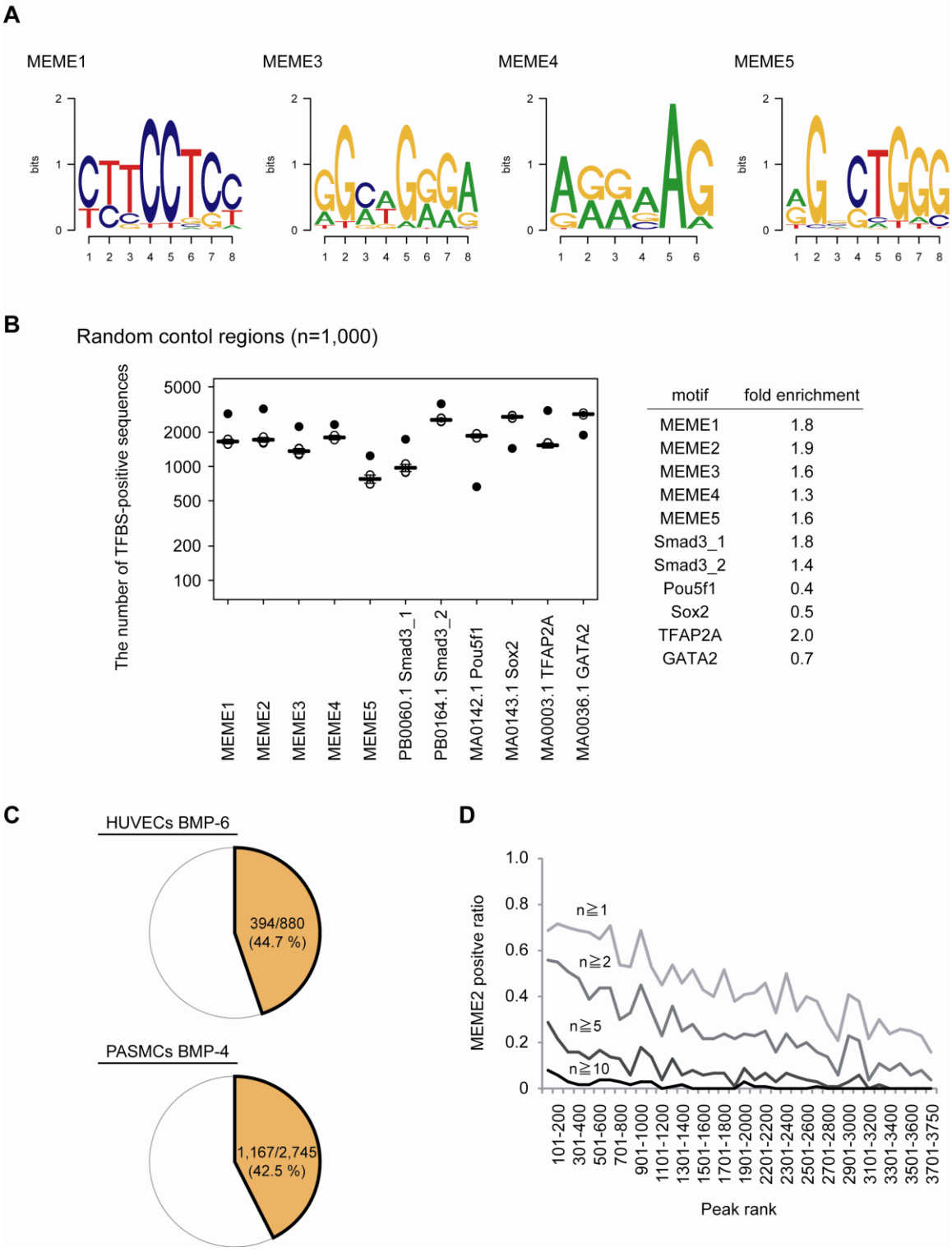
		Number	Ratio
2 h	counts	108	1
	bound	70	0.64
24 h	counts	318	1
	bound	116	0.36

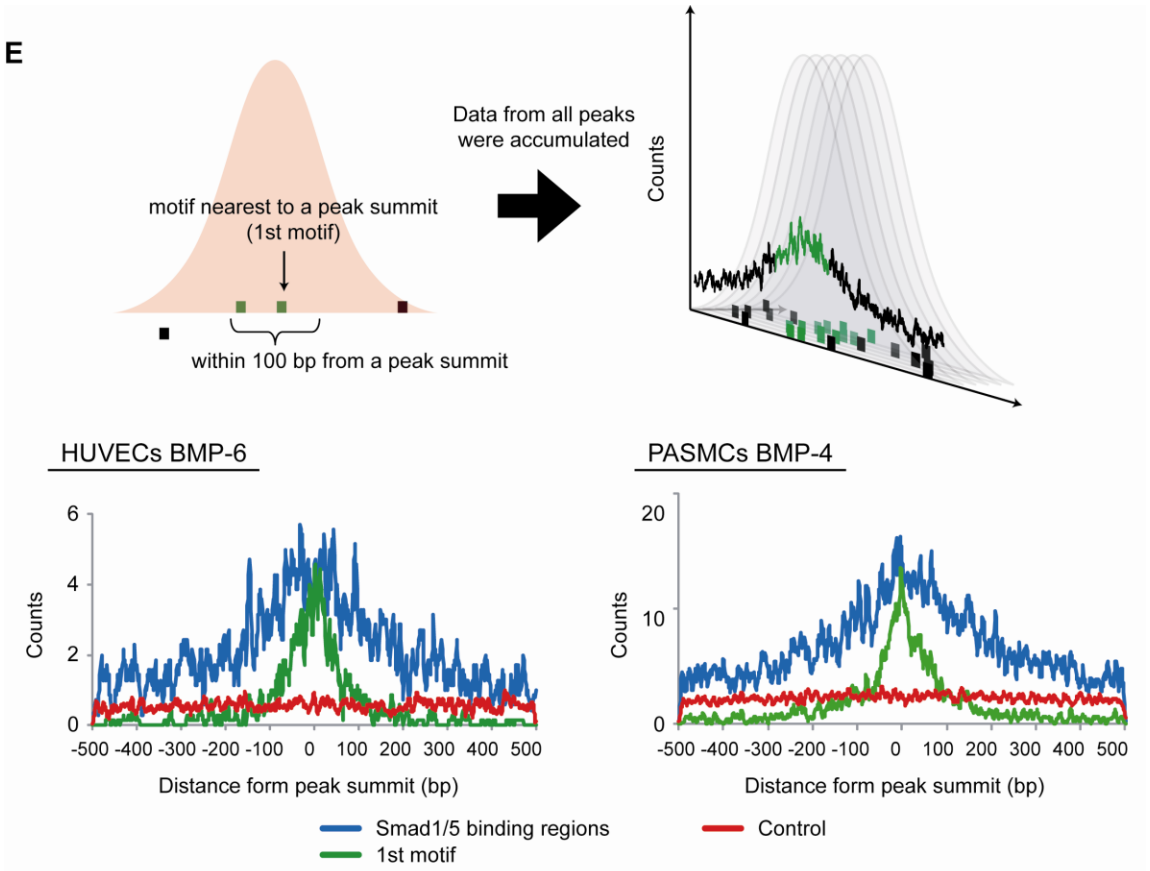
Down-regulated genes

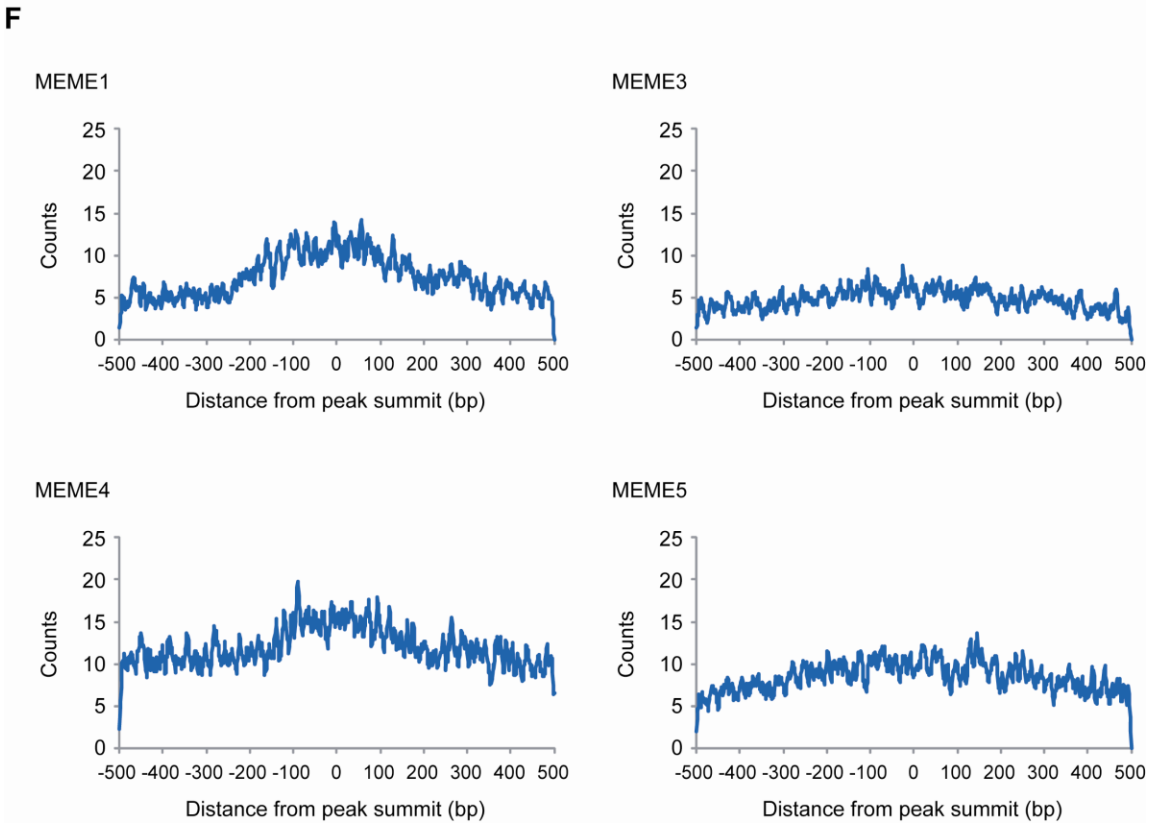
		Number	Ratio
2 h	counts	37	1
	bound	9	0.24
24 h	counts	264	1
	bound	58	0.22

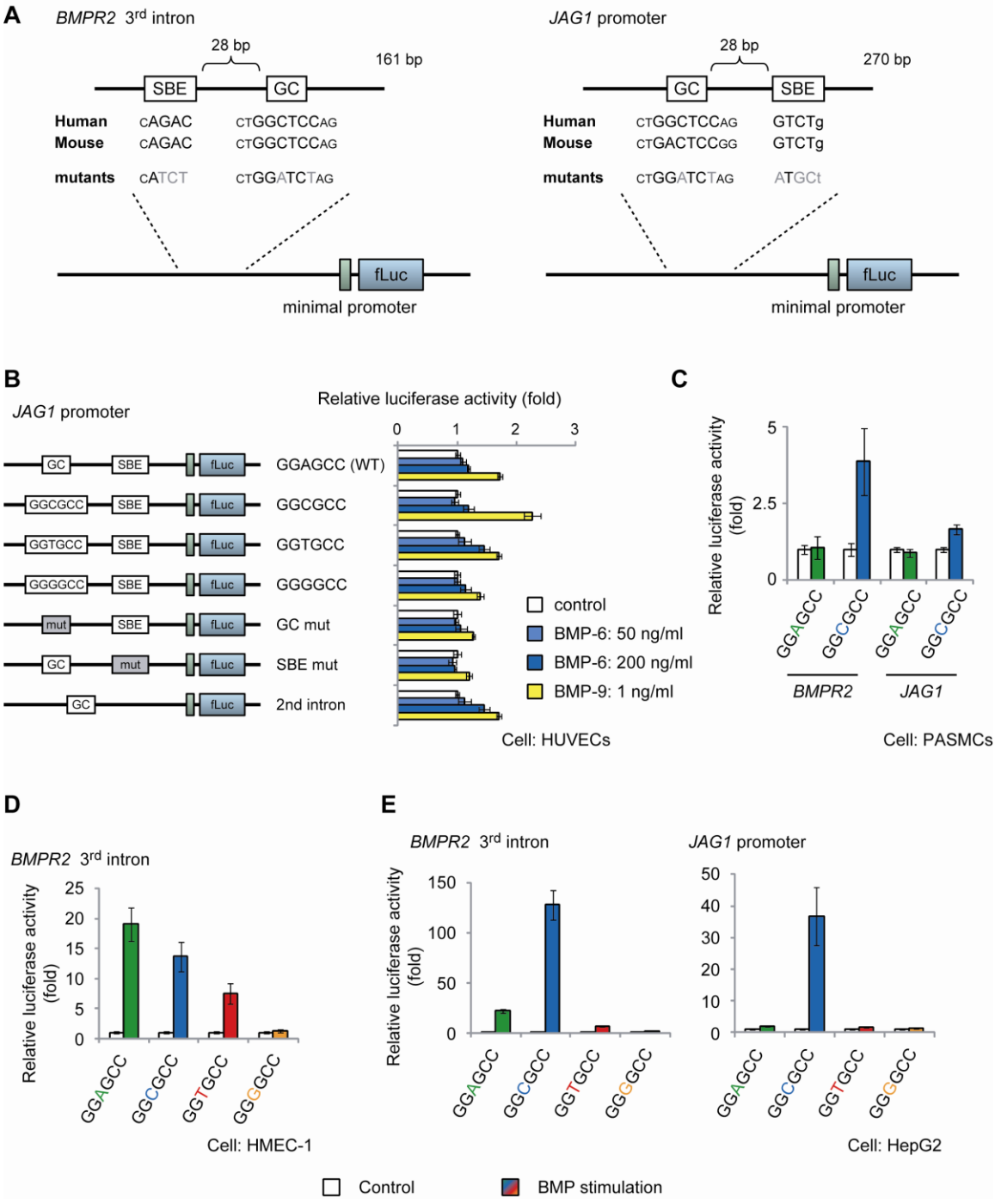
C

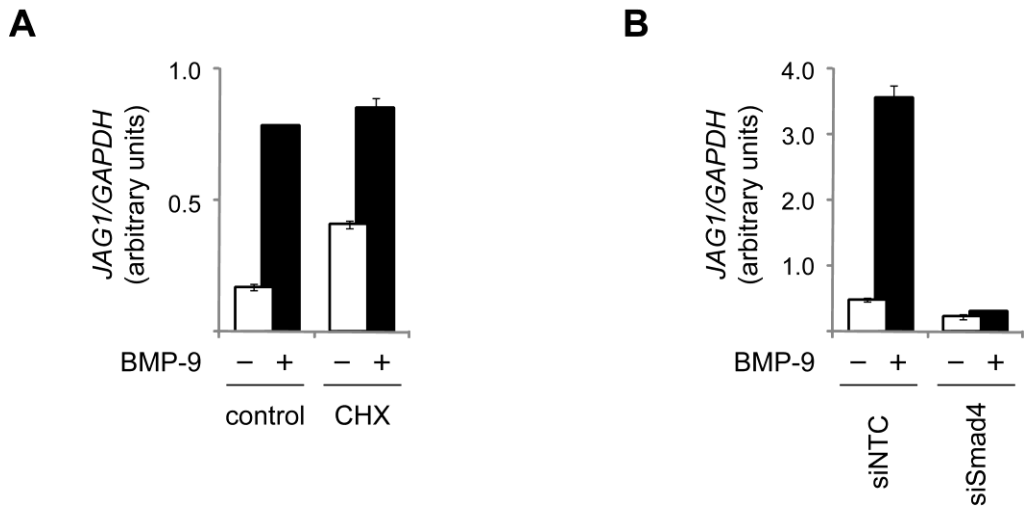



A
MEME1
MEME3
MEME4
MEME5
bits
B
Random contol regions (n=1,000)
The number of TFBS-positive sequences
5000
2000
1000
500
200
100
MEME1
MEME2
MEME3
MEME4
MEME5
PB0060.1 Smad3_1
PB0164.1 Smad3_2
MA0142.1 Pou5f1
MA0143.1 Sox2
MA0003.1 TFAP2A
MA0036.1 GATA2
motif fold enrichment
MEME1 1.8
MEME2 1.9
MEME3 1.6
MEME4 1.3
MEME5 1.6
Smad3_1 1.8
Smad3_2 1.4
Pou5f1 0.4
Sox2 0.5
TFAP2A 2.0
GATA2 0.7
C
HUVECs BMP-6
394/880
(44.7 %)
PASMCs BMP-4
1,167/2,745
(42.5 %)
D
MEME2 positive ratio
1.0
0.8
0.6
0.4
0.2
0
n≧1
n≧2
n≧5
n≧10
101-200
301-400
501-600
701-800
901-1000
1101-1200
1301-1400
1501-1600
1701-1800
1901-2000
2201-2200
2301-2400
2501-2600
2701-2800
2901-3000
3101-3200
3301-3400
3501-3600
3701-3750
Peak rank


E
motif nearest to a peak summit
(1st motif)
within 100 bp from a peak summit
Data from all peaks
were accumulated
Counts
HUVECs BMP-6
PASMCs BMP-4
Counts
Distance form peak summit (bp)
Smad1/5 binding regions
1st motif
Control


F
MEME1
MEME3
MEME4
MEME5
Counts
Distance from peak summit (bp)


A
BMPR2 3rd intron
28 bp
161 bp
SBE
GC
Human cAGAC ctGGCTCCag
Mouse cAGAC ctGGCTCCag
mutants cATCT ctGGATCTag
fLuc
minimal promoter
JAG1 promoter
28 bp
270 bp
GC
SBE
Human ctGGCTCCag GTCTg
Mouse ctGACTCCgg GTCTg
mutants ctGGATCTag ATGCt
fLuc
minimal promoter
B
JAG1 promoter
Relative luciferase activity (fold)
0 1 2 3
GC SBE fLuc GGAGCC (WT)
GGCGCC SBE fLuc GGCGCC
GGTGCC SBE fLuc GGTGCC
GGGGCC SBE fLuc GGGGCC
mut SBE fLuc GC mut
GC mut fLuc SBE mut
GC fLuc 2nd intron
control
BMP-6: 50 ng/ml
BMP-6: 200 ng/ml
BMP-9: 1 ng/ml
Cell: HUVECs
C
Relative luciferase activity (fold)
5
2.5
0
GGAGCC
GGCGCC
GGAGCC
GGCGCC
BMPR2
JAG1
Cell: PASMCs
D
BMPR2 3rd intron
Relative luciferase activity (fold)
25
20
15
10
5
0
GGAGCC
GGCGCC
GGTGCC
GGGGCC
Cell: HMEC-1
E
BMPR2 3rd intron
Relative luciferase activity (fold)
150
100
50
0
GGAGCC
GGCGCC
GGTGCC
GGGGCC
JAG1 promoter
50
40
30
20
10
0
GGAGCC
GGCGCC
GGTGCC
GGGGCC
Cell: HepG2
Control
BMP stimulation


A
JAG1/GAPDH (arbitrary units)
1.0
0.5
BMP-9 − + − +
control CHX
B
JAG1/GAPDH (arbitrary units)
4.0
3.0
2.0
1.0
BMP-9 − + − +
siNTC
siSmad4