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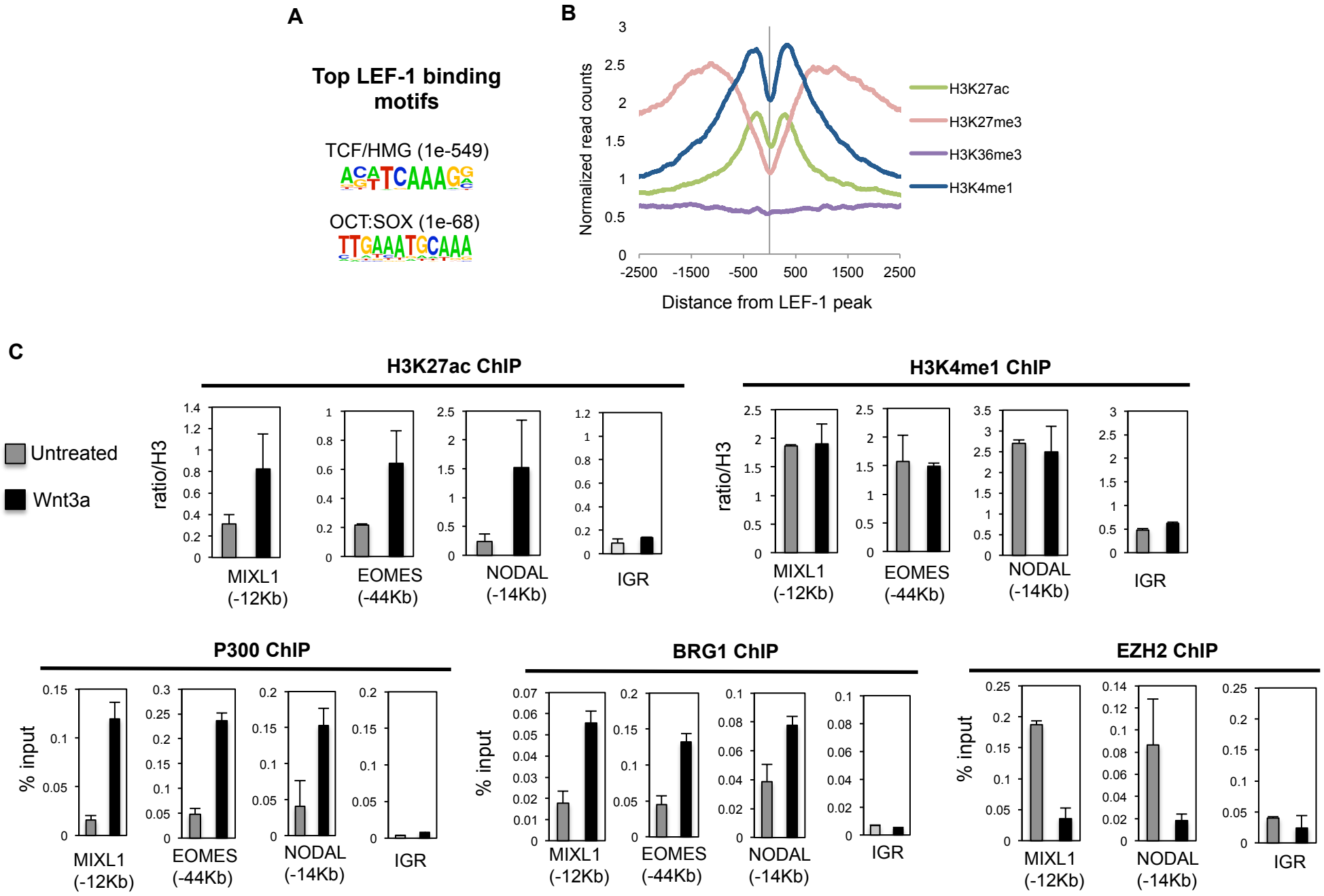
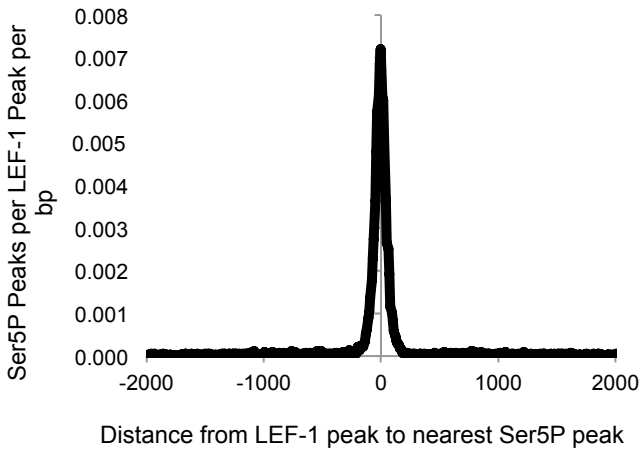
Supplemental Information

SMADs and YAP Compete to Control Elongation of β-Catenin:LEF-1-Recruited RNAPII during hESC Differentiation

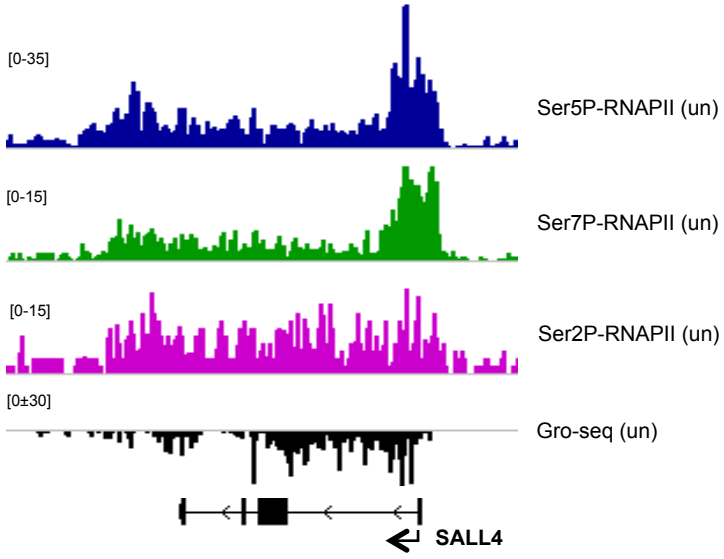
Conchi Estarás, Chris Benner, and Katherine A. Jones


A
Top LEF-1 binding motifs
TCF/HMG (1e-549)
OCT:SOX (1e-68)
B
Normalized read counts
Distance from LEF-1 peak
H3K27ac
H3K27me3
H3K36me3
H3K4me1
C
Untreated
Wnt3a
H3K27ac ChIP
H3K4me1 ChIP
P300 ChIP
BRG1 ChIP
EZH2 ChIP
ratio/H3
% input
MIXL1 (-12Kb)
EOMES (-44Kb)
NODAL (-14Kb)
IGR

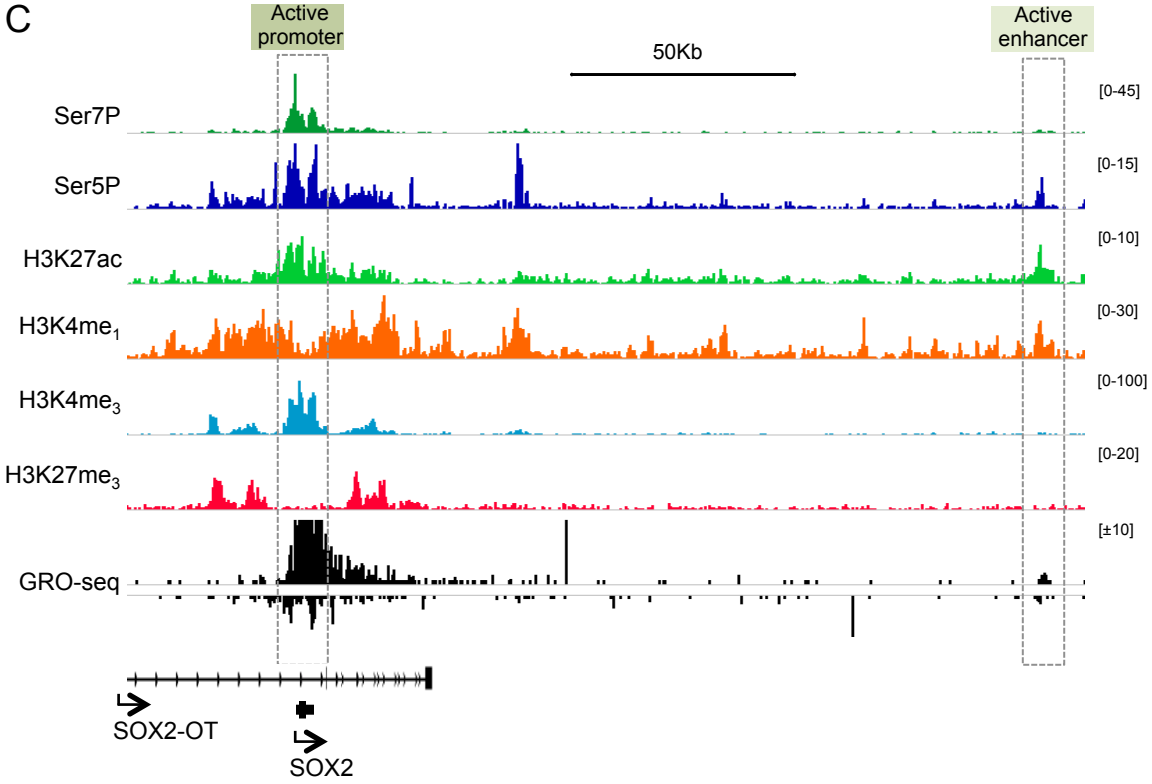
A



B



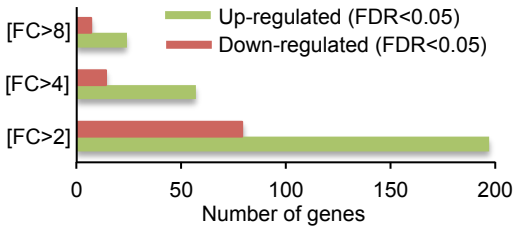
C



D

Ser5P and Ser7P RNAPII in Promoters and Enhancers	
Active enhancer ($K4me_1$: K27ac)	**Ser5P⬆ Ser7P⬇**
Poised enhancer ($K4me_1$: $K27me_3$)	**Ser5P⬆ Ser7P⬇**
Active promoter	**Ser5P⬆ Ser7P⬆**
Poised promoter	**Ser5P⬆ Ser7P⬇**
Inactive promoter	**Ser5P⬇ Ser7P⬇**

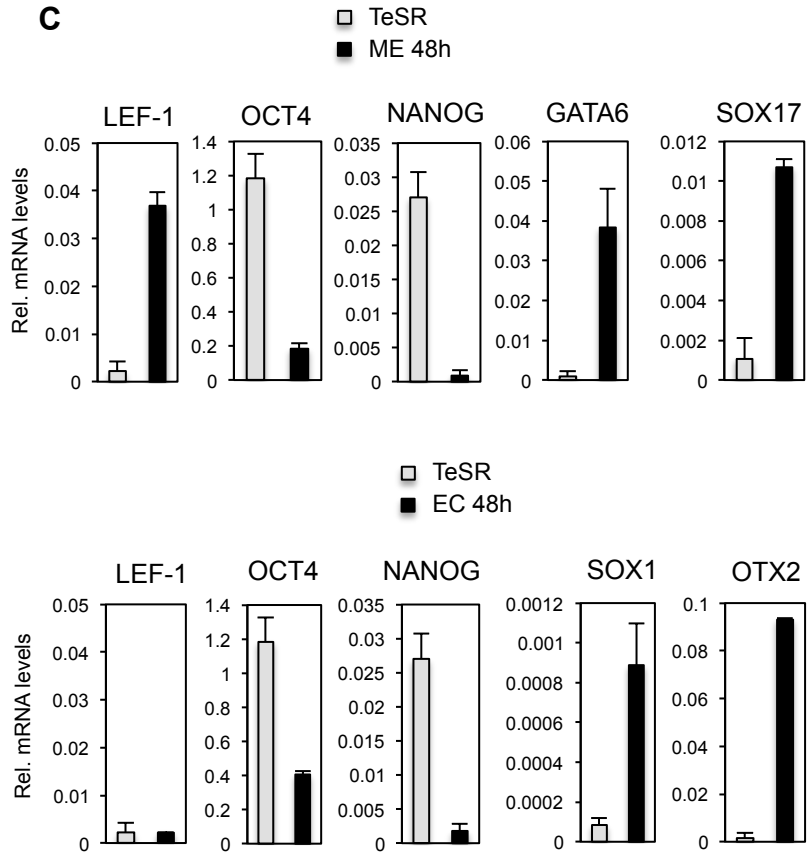
A

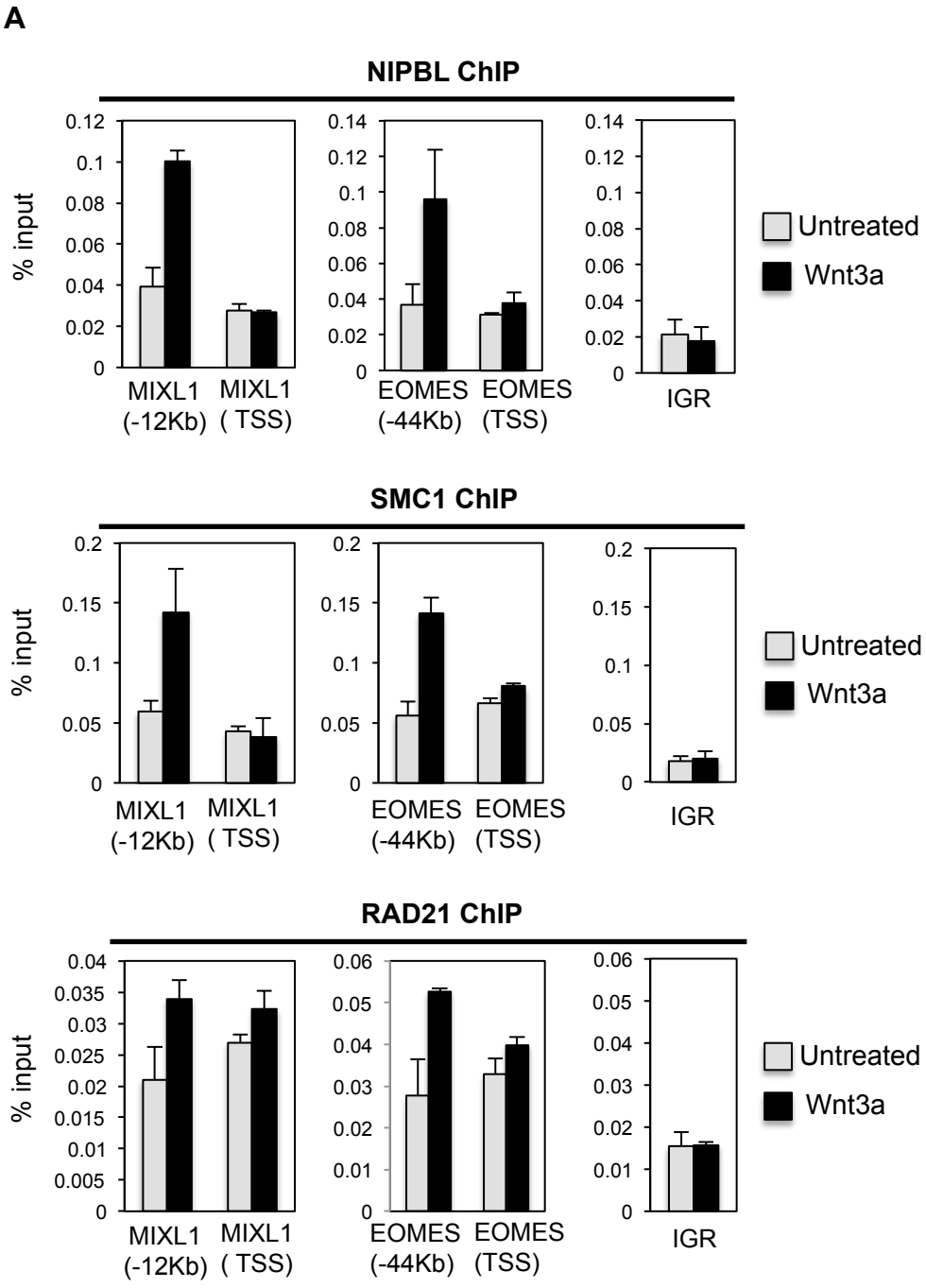


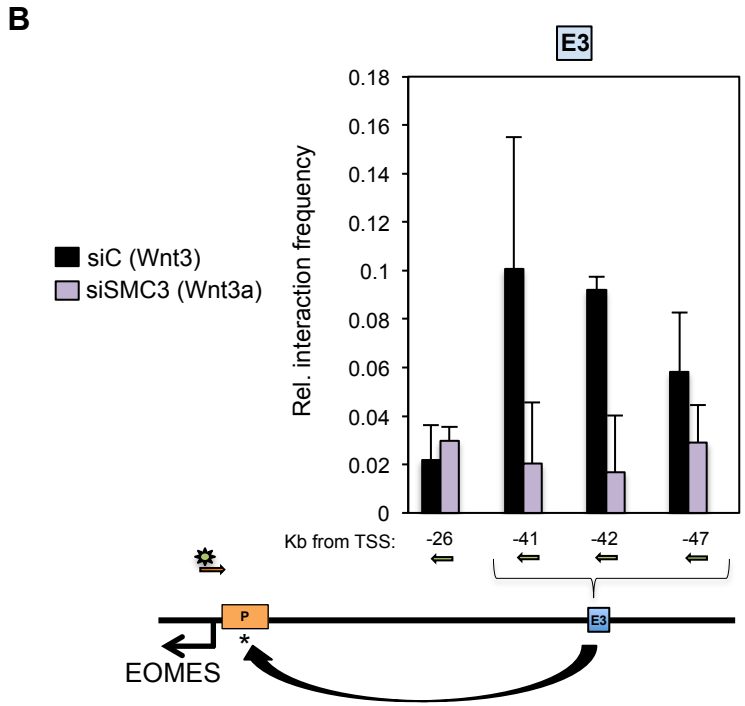
B

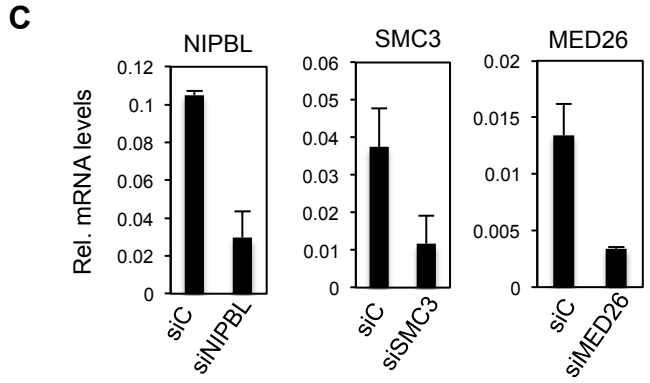
GO term (Wnt3a induced genes)	FDR
GO:0007275~multicellular organismal development	3.19E-07
GO:0032502~developmental process	1.50E-06
GO:0048731~system development	5.55E-06
GO:0014032~neural crest cell development	2.74E-05
GO:0014033~neural crest cell differentiation	2.74E-05
GO:0014031~mesenchymal cell development	2.92E-05
GO:0048762~mesenchymal cell differentiation	3.12E-05
GO:0060485~mesenchyme development	3.66E-05
GO:0001667~ameboidal cell migration	6.40E-05
GO:0001755~neural crest cell migration	7.52E-05
GO:0048856~anatomical structure development	8.35E-05
GO:0032501~multicellular organismal process	1.39E-04
GO:0030154~cell differentiation	5.22E-04
GO:0007399~nervous system development	6.98E-04
GO:0048869~cellular developmental process	0.001429587
GO:0005576~extracellular region	0.005368669
GO:0009887~organ morphogenesis	0.005949419
GO:0016055~Wnt receptor signaling pathway	0.006154679
GO:0005576~extracellular region	0.006244501
GO:0048513~organ development	0.00629155
GO:0044421~extracellular region part	0.008916906
GO:0031012~extracellular matrix	0.011001359
GO:0031012~extracellular matrix	0.013161438
GO:0016477~cell migration	0.013756399
GO:0016477~cell migration	0.014989624
GO:0009888~tissue development	0.015374614
GO:0005578~proteinaceous extracellular matrix	0.025934456
GO:0009790~embryonic development	0.02625073
GO:0005578~proteinaceous extracellular matrix	0.030762369
hsa05217:Basal cell carcinoma	0.034980386
GO:0048468~cell development	0.036297817
GO:0048870~cell motility	0.03982481
GO:0051674~localization of cell	0.043306283
GO:0048870~cell motility	0.043306283
hsa04310:Wnt signaling pathway	0.047720187

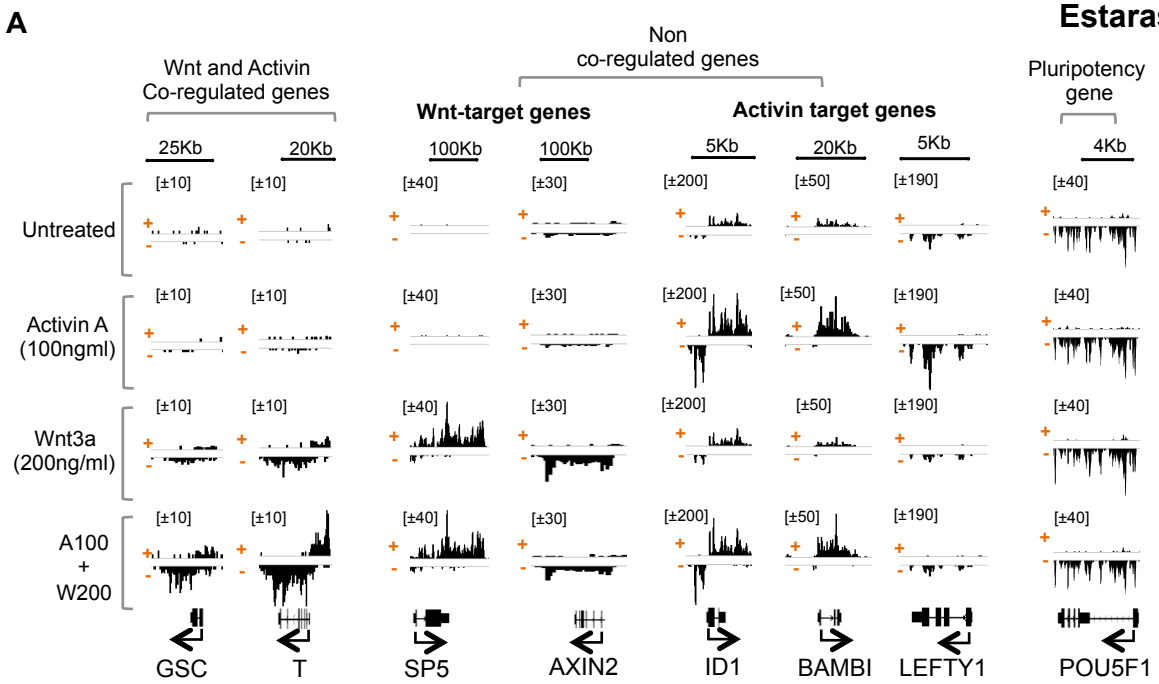
C




A
NIPBL ChIP
% input
0.12
0.1
0.08
0.06
0.04
0.02
0
MIXL1 (-12Kb)
MIXL1 (TSS)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
EOMES (-44Kb)
EOMES (TSS)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
IGR
Untreated
Wnt3a
SMC1 ChIP
% input
0.2
0.15
0.1
0.05
0
MIXL1 (-12Kb)
MIXL1 (TSS)
0.2
0.15
0.1
0.05
0
EOMES (-44Kb)
EOMES (TSS)
0.2
0.15
0.1
0.05
0
IGR
Untreated
Wnt3a
RAD21 ChIP
% input
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
MIXL1 (-12Kb)
MIXL1 (TSS)
0.06
0.05
0.04
0.03
0.02
0.01
0
EOMES (-44Kb)
EOMES (TSS)
0.06
0.05
0.04
0.03
0.02
0.01
0
IGR
Untreated
Wnt3a

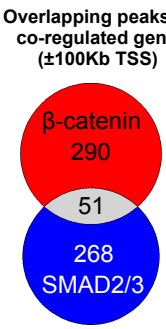

B
E3
siC (Wnt3)
siSMC3 (Wnt3a)
Rel. interaction frequency
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Kb from TSS:
-26
-41
-42
-47
P
E3
*
EOMES

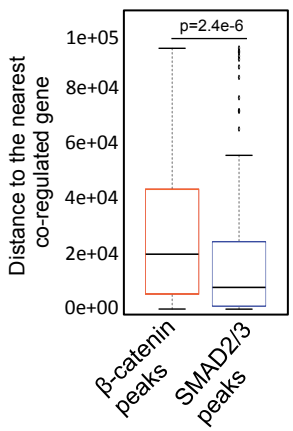

C
NIPBL
SMC3
MED26
Rel. mRNA levels
0.12
0.1
0.08
0.06
0.04
0.02
0
0.06
0.05
0.04
0.03
0.02
0.01
0
0.02
0.015
0.01
0.005
0
siC
siNIPBL
siC
siSMC3
siC
siMED26

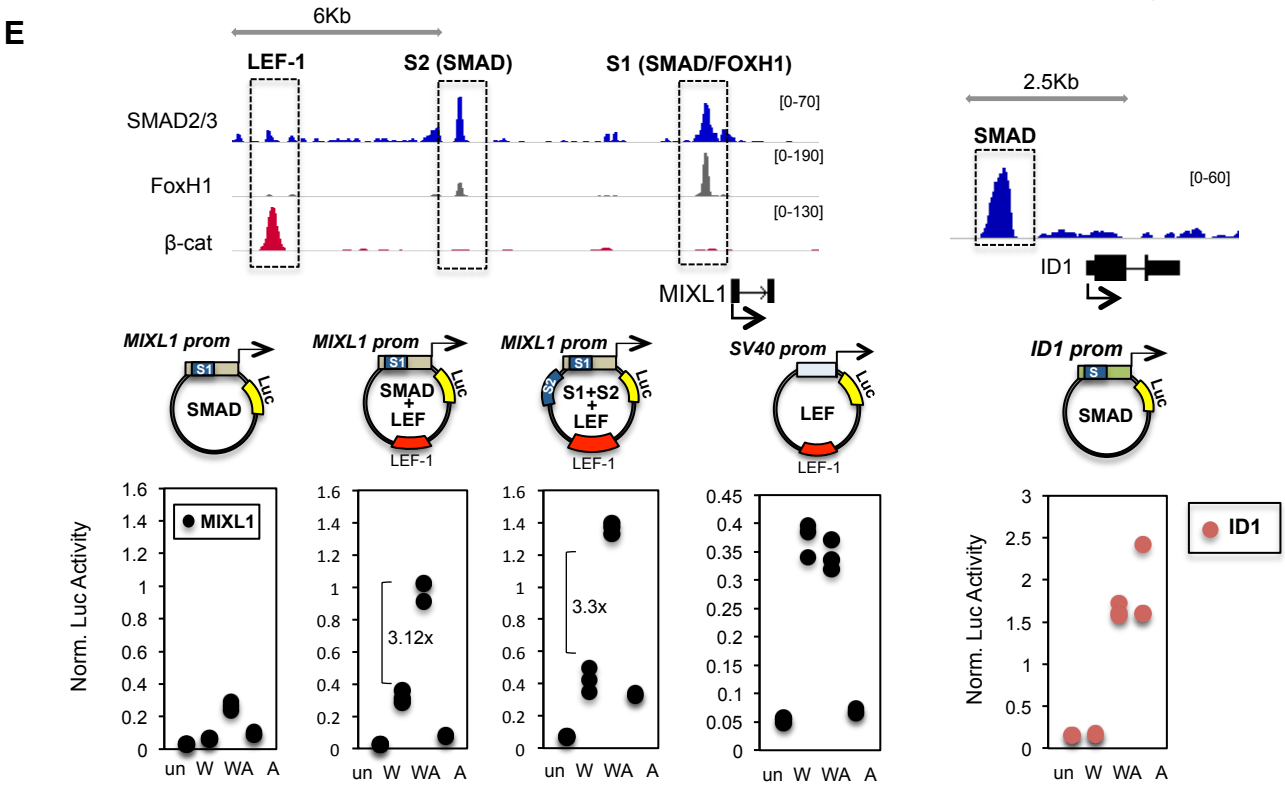


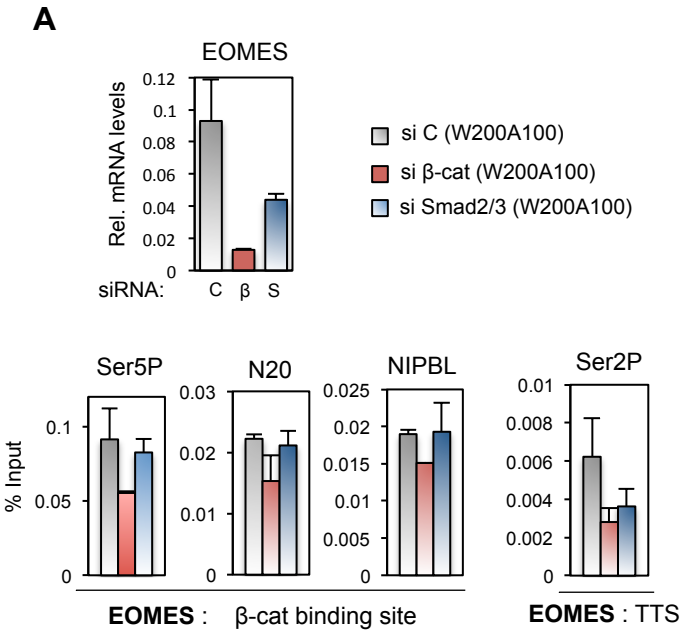
B

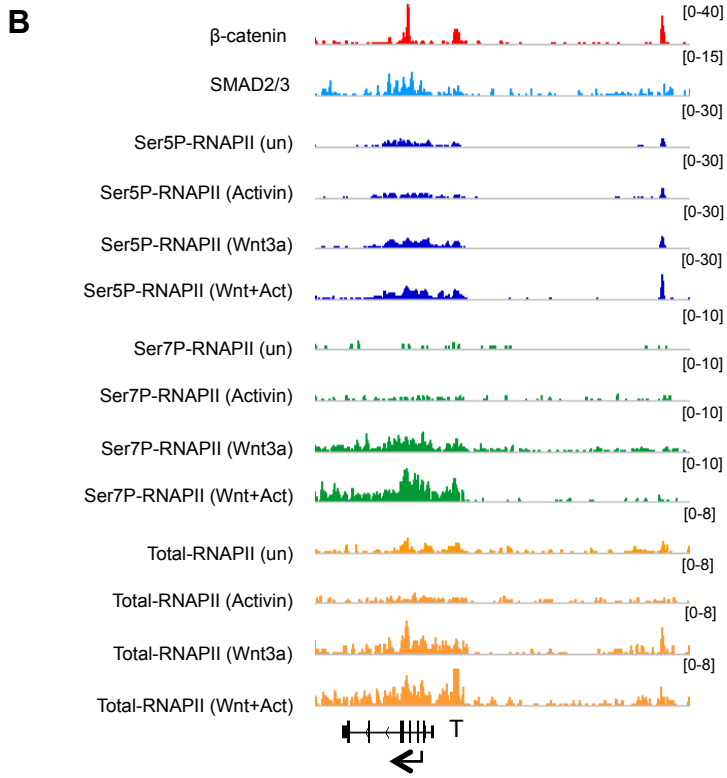
GO description of co-regulated genes	FDR
Multicellular org.development	1.35-E^{-06}
System development	3.03-E^{-05}
Anat. struct. dev.	3.04-E^{-05}
Developmental process	9.56-E^{-05}
Organ development	2.60-E^{-02}
Pattern specif. process	6.99-E^{-02}
Glycosylation site:N-linked	2.05-E^{-02}

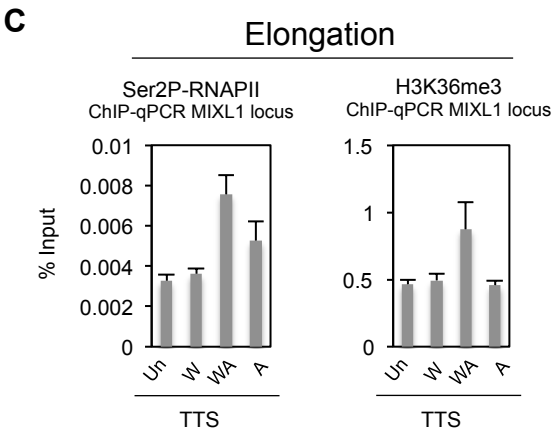


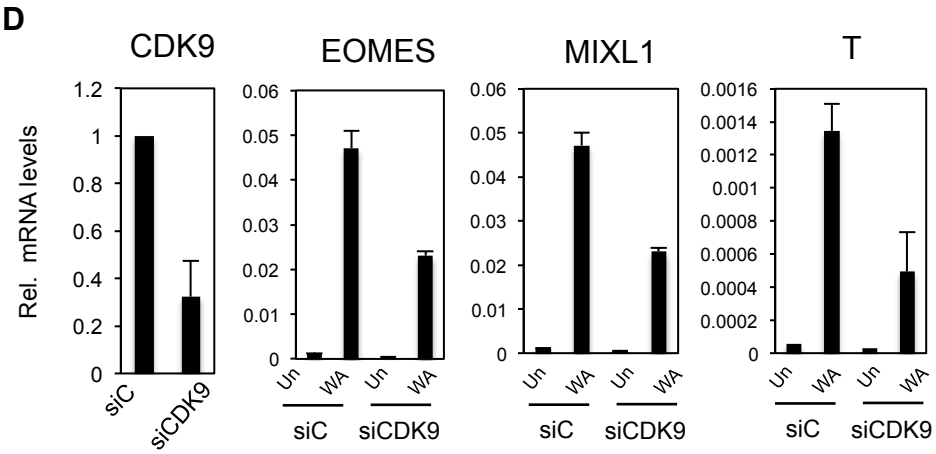


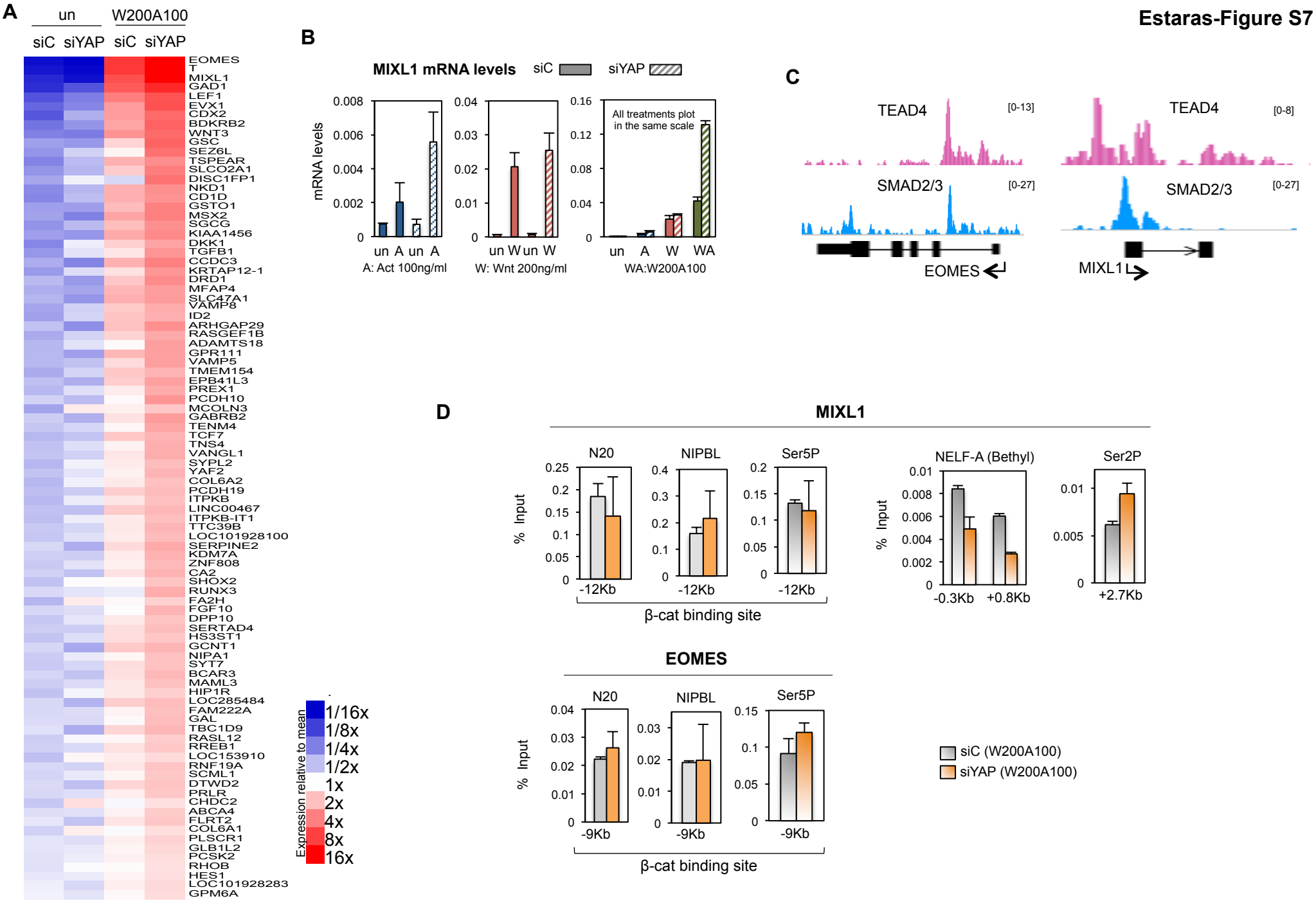



A
EOMES
Rel. mRNA levels
0.12
0.1
0.08
0.06
0.04
0.02
0
siRNA: C β S
si C (W200A100)
si β-cat (W200A100)
si Smad2/3 (W200A100)
Ser5P
N20
NIPBL
Ser2P
% Input
EOMES : β-cat binding site
EOMES : TTS


B
β-catenin
SMAD2/3
Ser5P-RNAPII (un)
Ser5P-RNAPII (Activin)
Ser5P-RNAPII (Wnt3a)
Ser5P-RNAPII (Wnt+Act)
Ser7P-RNAPII (un)
Ser7P-RNAPII (Activin)
Ser7P-RNAPII (Wnt3a)
Ser7P-RNAPII (Wnt+Act)
Total-RNAPII (un)
Total-RNAPII (Activin)
Total-RNAPII (Wnt3a)
Total-RNAPII (Wnt+Act)
[0-40]
[0-15]
[0-30]
[0-10]
[0-8]
T


C
Elongation
Ser2P-RNAPII
ChIP-qPCR MIXL1 locus
H3K36me3
ChIP-qPCR MIXL1 locus
% Input
Un W WA A
TTS


D
CDK9
EOMES
MIXL1
T
Rel. mRNA levels
siC
siCDK9
Un WA Un WA


A
un
W200A100
siC siYAP siC siYAP
EOMES
T
MIXL1
GAD1
LEF1
EVX1
CDX2
BDKRB2
WNT3
GSC
SEZ6L
TSPEAR
SLCO2A1
DISC1FP1
NKD1
CD1D
GSTO1
MSX2
SGCG
KIAA1456
DKK1
TGFB1
CCDC3
KRTAP12-1
DRD1
MFAP4
SLC47A1
VAMP8
ID2
ARHGAP29
RASGEF1B
ADAMTS18
GPR111
VAMP5
TMEM154
EPB41L3
PREX1
PCDH10
MCOLN3
GABRB2
TENM4
TCF7
TNS4
VANGL1
SYPL2
YAF2
COL6A2
PCDH19
ITPKB
LINC00467
ITPKB-IT1
TTC39B
LOC101928100
SERPINE2
KDM7A
ZNF808
CA2
SHOX2
RUNX3
FA2H
FGF10
DPP10
SERTAD4
HS3ST1
GCNT1
NIPA1
SYT7
BCAR3
MAML3
HIP1R
LOC285484
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GAL
TBC1D9
RASL12
RREB1
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RNF19A
SCML1
DTWD2
PRLR
CHDC2
ABCA4
FLRT2
COL6A1
PLSCR1
GLB1L2
PCSK2
RHOB
HES1
LOC101928283
GPM6A
Expression relative to mean
1/16x
1/8x
1/4x
1/2x
1x
2x
4x
8x
16x
B
MIXL1 mRNA levels
siC
siYAP
mRNA levels
0.008
0.006
0.004
0.002
0
un A un A
A: Act 100ng/ml
0.04
0.03
0.02
0.01
0
un W un W
W: Wnt 200ng/ml
0.16
0.12
0.08
0.04
0
All treatments plot in the same scale
un A W WA
WA:W200A100
C
TEAD4
[0-13]
TEAD4
[0-8]
SMAD2/3
[0-27]
SMAD2/3
[0-27]
EOMES
MIXL1
D
MIXL1
N20
NIPBL
Ser5P
% Input
0.25
0.2
0.15
0.1
0.05
0
0.4
0.3
0.2
0.1
0
-12Kb
β-cat binding site
NELF-A (Bethyl)
Ser2P
0.01
0.008
0.006
0.004
0.002
0
-0.3Kb
+0.8Kb
0.005
+2.7Kb
EOMES
N20
NIPBL
Ser5P
0.04
0.03
0.02
0.01
0
0.15
0.1
0.05
-9Kb
β-cat binding site
siC (W200A100)
siYAP (W200A100)

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. Analysis of β-catenin binding to LEF-1 enhancers in Wnt3a-signaling hESCs. (A) Top LEF-1 binding motifs in H1 hESCs identified by ChIP-seq. (B) Histogram profile of histone modifications at LEF-1 binding sites genomewide. (C) ChIP-qPCR analysis at ME genes in untreated and Wnt3a treated hESCs (4h). The antibodies used are shown above each panel, and the genes analyzed are indicated at the bottom. Mean (SD; n=2).

Figure S2, related to Figure 2. RNAPII complexes at LEF-1 sites and hESC enhancers are enriched in CTD-Ser5P, but not -Ser7P. (A) Metaprofile showing the correlation between Ser5P-RNAPII and LEF-1 peaks in self-renewal H1 hESCs. (B) Capture showing ChIP-seq analysis of RNAPII -Ser5P, -Ser7P and -Ser2P and GRO-seq profiles at the active *SALL4* gene in untreated hESCs. (C) Genome browser capture shows examples of the ChIP-seq profile at the pluripotency gene, *SOX2*. Specific antibodies used are shown at the left. Scale bar and gene diagram are depicted above and below the capture, respectively. The dashed boxes highlight active enhancer and promoter regions, as indicated at the top. (D) Table summarizing the data in Figure 2. Note that enhancers are enriched in Ser5P-RNAPII, with low Ser7P, regardless their activation status.

Figure S3, related to Figure 3. GRO-seq analysis of Wnt3a-induced genes in hESCs. (A) Diagram indicating the number of genes induced (green) or repressed (red) in response to Wnt3a (200ng/ml, t=6h) in hESC GRO-seq experiments. Absolute fold change ([FC]) is shown in the vertical axis. (B) List of GO categories associated with Wnt3a-induced genes in hESCs. (C) hESCs were differentiated to mesoendoderm (ME; top panel) or ectoderm (EC; bottom panel) progenitors (see Experimental Procedures) for two days. Graphs show relative mRNA levels of the *LEF-1* gene, as well as lineage-specific marker genes for the ME (upper graph) and EC (lower graph) precursor cells. Mean (SD; n=2).

Figure S4, related to Figure 4. β-catenin recruits cohesin and induces long-range enhancer-promoter looping in Wnt3a-signaling hESCs. (A) ChIP-qPCR analysis of the recruitment of different cohesin subunits (SMC1,NIPBL and RAD21) before and after Wnt3a treatment (4h) at the *EOMES* and *MIXL1* gene enhancer (enh), promoter (TSS) or a negative control region (IGR). Mean (SD; n=2). (B) A 3C analysis of enhancer-promoter looping in Wnt3a treated hESCs that had been previously transfected with control or SMC3-specific siRNAs. The *EOMES* E3 enhancer (-40Kb from TSS) was analyzed by qPCR. Mean (SD; n=2). (C) qPCR analysis shows that the indicated siRNA treatments affect the mRNA levels of *NIPBL*, *SMC3* and *MED26* genes. Mean (SD; n=2).

Figure S5, related to Figure 5. Analysis of Wnt-Activin synergy at ME differentiation genes. (A) Genome browser capture examples of the GRO-seq profile for the Wnt-Activin co-regulated genes *GSC* and *T/Brachyury,* along with Wnt-specific (*AXIN2* and *SP5*) and Activin-specific target genes (*LEFTY*, *ID1*, *BAMBI*). Note that

combined Wnt3a-Activin A treatment only potentiates the developmental, but not canonical, target genes. For comparison, the GRO-seq profile of the active pluripotent *POU5F1* gene is shown on the right. (B) GO categories of 189 identified Wnt3a-Activin A co-regulated genes. (C) Venn diagram showing the overlap of SMAD2,3 and β-catenin peaks within 100Kb of the TSS for the 189 co-regulated genes. (D) Box diagram indicating the distance of SMAD2,3 and β-catenin peaks to the TSS of the nearest co-regulated gene. (E) Graph shows normalized luciferase activity of transfected hESCs treated as indicated below the graph (9h). The MIXL1 gene regulatory regions were assessed (see capture on top) and four different constructs were tested: 1) SMAD plasmid, which carries SMAD/FOXH1 (-0.3 Kb) element, 2) SMAD+LEF plasmid, which contains the SMAD/FOXH1 promoter element and LEF-1 (-12Kb) sites; 3) SMAD+LEF+S2, which contains the additional SMAD site at -5Kb from TSS and 4) LEF plasmid, which contains the LEF-1 enhancer site and the heterologous SV40 promoter, which lacks SMAD binding sites. On the right, the SMAD site from the ID1 gene was used as a control for Activin A signaling in these assays. The graphs plot Luciferase activity normalized to Renilla from three independent biological replicates.

Figure S6, related to Figure 6. Activin/SMAD2,3 signaling stimulates P-TEFb elongation at ME genes. (A) siRNA-ChIPs in β-catenin- and SMAD2,3-depleted hESCs. Following siRNA transfection (48h), cells were treated with Wnt3a plus Activin A (W200A100, 4h) prior to qPCR-ChIP analysis of the *EOMES* gene, using the antibodies indicated in the legend. The positions of the primers are indicated in the gene diagram. Graph on top show mRNA levels of *EOMES* in the siRNA treated cells. (B) Genome browser capture showing ChIP-seq profiles of *T/Brachyury* regulatory regions in untreated, Wnt3a, Activin A and Wnt+Activin treated cells. Legend on the left shows the IP antibody used and the specific treatment in parenthesis. Gene diagrams are depicted at the bottom. (C) ChIP-qPCR showing Ser2P-RNAPII and H3K36me3 levels at *MIXL1* TTS in response to the indicated treatments (below the graph). (D) qPCR analysis shows mRNA levels of *MIXL1*, *EOMES* and *T* genes in response to Wnt3a+Activin in control (siC) and CDK9-depleted cells (siCDK9). Graph on the left shows the extent to which the siRNA depletion affected endogenous CDK9 mRNA levels. Mean (SD; n=2).

Figure S7, related to Figure 7. Wnt-Activin synergy overcomes YAP inhibition of elongation at ME genes. (A) Heatmap showing the expression levels of the 89 Wnt3a+Activin co-regulated genes affected by YAP depletion in H1 hESCs. (B) Analysis of *MIXL1* mRNA levels in YAP-depleted (dashed) or control (solid) hESCs treated with Activin A alone (blue), Wnt3a alone (red) or both cytokines (green). Mean +SD (n=2). Note that different scales are used for the three graphs, and that the graph at right shows the response to the 3 treatments plotted at the same scale. (C) Genome browser capture showing TEAD4 and SMAD2,3 co-localization on *MIXL1* and *EOMES* promoters. (D) siRNA-ChIP in YAP-depleted hESCs. Following siRNA transfection (48h), cells were treated with Wnt3a plus Activin A (W200A100, 4h) prior to qPCR-ChIP analysis of the *MIXL1* and *EOMES* genes, using the antibodies indicated in the legend. The location of the primers are indicated below the graphs. Mean (SD; n=2).

Table S1, related to Figure 5. Wnt+Activin co-regulated genes in hESCs. GRO-seq experiment in untreated, Wnt3a, Activin A and Wnt3a+Activin treated cells (t=6h). The table contains the list of 189 Wnt+Activin co-regulated genes. The co-regulated genes

are defined as those genes more active in the co-treatment condition than in any single treatment (FC>1.25). The columns are grouped as indicated: grey header; transcripts ID, yellow header; raw GRO-seq reads in each treatment, purple header; Activin-induced Fold Change, p value and adjusted p value, orange header; Wnt-induced Fold Change, p value and adjusted p value, blue header; Wnt+Activin-induced Fold Change, p value and adjusted p value.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

hESCs culture

H1 hESCs were cultured in mTeSR1 on Matrigel-coated (BD Biosciences) tissue culture plates. hESCs colonies were expanded manually at 1:3 split ratio every 5–7 d. Medium was replaced daily. For experiments, clumps were disaggregated into single cells at 1:6 split ratio following treatment with Accutase in presence of Rock inhibitors. Mouse recombinant Wnt3a (R&D 5036-WN-010), Activin A (Millipore GF300) and GSK3 inhibitor XV (Millipore 361558) was added to the mTeSR1 medium, unless otherwise indicated. Note: in our experience, different lots of these cytokines exhibit variation in their bioactivity.

Early Ectoderm differentiation

hESCs colonies were disaggregated into single cells using accutase. hESCs were allowed to expand in mTeSR1 medium until they were nearly confluent (~3 days). The initial differentiation media contained knockout serum replacement media with 10 μM TGF-beta inhibitor (SB431542, Millipore) and 200 ng/ml of Noggin (R&D). Cell media was replaced every day. Cells were collected following 2nd and 3rd day of differentiation and expression of early ectoderm markers were analyzed.

Mesendoderm differentiation

For differentiation into ME lineages, hESCs clumps were disaggregated with accutase and cultured in matrigel-coated plates on mTeSR1 media. When cells reached sub-confluence, Activin A and Wnt3a (or GSK3 inhibitor XV) were added to the mTeSR1 media at indicated concentrations. Media and cytokines were replaced every day. ME markers were analyzed 24 and 48 after differentiation induction.

mRNA extraction and Quantitative PCR

Total RNA was extracted using Trizol reagent (Life Technologies) following manufacturer indications. Then, 1 μg of total RNA was reverse transcribed using Transcriptor First Strand Synthesis kit (Roche). Amplified material was detected using SYBR green master mix (Life Technologies) on an ABI7300 (Applied Biosystems) thermo-cycler. All results were normalized to a RPS23 gene control.

siRNA transfection

hESCs were transfected with Lipofectamine RNAiMAX transfection reagent by following instructions from the supplier. Assays were performed 24-48 h after transfection, as indicated.

Chromatin Conformation Capture (3C) assays

Chromatin conformation capture (3C) assays were carried out according to Gavrilov et al., 2009 with minor modifications. hESCs were disaggregated using accutase. When cells were nearly confluent, cells were crosslinked with 2% formaldehyde for 15 minutes at room temperature. The reaction was quenched with 125 mM glycine. After two washes with PBS1x, cells were lysed with 15 mL of ice cold lysis buffer (10 mM Tris-HCl

pH 8.0, 10 mM NaCl, 0.2% NP-40 and 1x complete proteinase inhibitors (Roche)). Following 20 minutes incubation on ice, nuclei were pelleted and resuspended in 0.5 mL of 3.1 restriction buffer (NEB) to 1.2x final concentration. Then, nuclei were incubated with vigorous shaking at 37°C, in presence of SDS 0.3%. Next, Triton X-100 was added at a final concentration of 1.8% and samples were placed in a shaker at 37°C for an additional 1h. DNA digestion was then carried out overnight at 37°C following addition of 1000 units of *BglII* or *BamHI* (for *EOMES* or *MIXL1* locus analysis, respectively). After enzyme inactivation (by addition of 1.3% SDS at 65°C, 20min) samples were incubated with 7 mL of 1x ligation buffer and 1% final concentration of Triton X-100, for 1 hour at 37°C. Then, 100 Units of T4 DNA ligase (Fermentas) were added and samples were incubated at 16°C overnight with slow agitation. To reverse cross-linking, DNA was incubated overnight at 65°C. Finally, DNA was phenol/chloroform extracted and precipitated with ethanol. BAC clone RP11-816O9 containing human *MIXL1* locus and BAC clone RP11-626E24 containing human *EOMES* locus were used as controls. Ten µg of the BAC clone were digested with 500 Units of *BamHI* (*MIXL1*) or *BglII* (*EOMES*) overnight at 37°C with agitation. Following phenol-chloroform extraction, DNA fragments were ligated with 50 Units of T4 DNA ligase at 16°C overnight with slow agitation. The ligated BAC DNA was then extracted with phenol/chloroform and precipitated with ethanol. Taqman probes flanking the *EOMES* promoter region and *MIXL1* -12Kb enhancer (M1), along with the primers were designed using Primer Express 3.0 (Applied Biosystems).

All primers were designed to anneal at and face the same ends of the *BglII* or *BamHI* sites, have melting temperatures of 58-60 °C and yield amplicons of 100-150 bp. Serial dilutions of the BAC DNA were used to generate the calibration curves and trendline equations. Primer pair efficiency was corrected using the corresponding equation in each qPCR. Amplification was performed using ABI7300 with initial denaturation at 95°C for 3 minutes, followed by 50 cycles of 94°C for 5 seconds and 60°C for 20 seconds. The interaction frequencies were calculated based on the amount of PCR products obtained from the anchor probe primer with each test primer pair compared to the control region. The control region spans from the anchor probe primer and a reverse primer inside the same restriction fragment. This control meant to be the positive control and to correct the total DNA in each sample. Normalized qPCR values were represented.

Sample treatments for ChIP-seq GRO-seq experiments.

hESCs clumps were disaggregated into single cells using Accutase and plated in 10cm^2 dishes. When cells reached ~80% confluence, Wnt3a (200ng/ml), Activin A (100ng/ml), or combination of both Wnt3a+Activin A (W200A100) cytokines were added to the mTeSR1 media for the specified times.

For GRO-seq in YAP depleted cells: hESCs clumps were disaggregated into single cells using Accutase and plated in 10cm^2 dishes. The following day, cells were transfected using RNAimax (Invitrogen) with scramble (siC) or YAP specific siRNAs (siYAP). After 40h, transfected cells were treated with Wnt3a +Activin A (W200A100) or left untreated for 6 additional hours. Subsequently, cells were processed as indicated in the ChIP-seq and GRO-seq procedures sections (see below).

ChIP-qPCR and ChIP-seq procedure

For ChIP experiments, approximately ~3×10^6 hESCs were double-crosslinked in plate. First, 0.2mM of di (N-succinimidyl) glutarate (DSG, Sigma, 80424) was added for 45 minutes at room temperature. Following two washes with PBS 1x, cells were incubated 20 min with 1% formaldehyde. Then cells were lysate in lysis-IP buffer and sonicated (4x60'', amp 3). After precipitating the non sonicated chromatin, cell extract was pre-cleared with protein A/G at 4C, 1h. Aprox. ~500ug of total protein was used for IP. After addition of 5-10 ug of antibody cell extracts were incubated overnight at 4°C. After four washes and reverse crosslinking, DNA was purified using Qiaquick PCR purification kit (Qiagen, 28106).For ChIP-qPCR, DNA was eluted in 75ul and 2ul was used for each qPCR reaction. Amplification was performed using SYBR Green master mix and was carried out in an ABI7300 device. Buffers: Lysis/IP buffer: 0.1% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 20 mM Tris pH 8, add fresh protease inhibitors. Wash buffer 1: 0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 20 mM HEPES. Wash buffer 2: 0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 20 mM HEPES. Wash buffer 3: 0.25 M LiCl, 0.5% NaDOC, 0.5% NP-40, 1 mM EDTA, 20 mM HEPES. Wash buffer 4: 1 mM EDTA, 20 mM HEPES. Elution buffer: 1% SDS, 0.1 M $NaHCO_3$.

The ChIP-seq procedure was essentially as described for ChIP-qPCR, with minor modifications. Two independent IP were performed for each condition and the DNA from the two replicates was pooled after the final elution. At least 5ng of total DNA was used to generate the libraries. ChIP DNA was end-repaired and 5' phosphorylated using T4 DNA Polymerase, Klenow and T4 Polynucleotide Kinase (Enzymatics). A single Adenine was added to 3' ends by Klenow (3-->5' exo-), and double-stranded Bioo Illumina Adapters (Bioo Scientific) were ligated to the ends of the ChIP fragments. Adapter-ligated ChIP DNA fragments were subjected to 15 cycles of PCR amplification using Q5 polymerase (NEB). AMPure beads were used to purify DNA after each step (Beckman Coulter). Library preparation and high-throughput sequencing using HiSeq 2500 device were carried out at Next Generation Sequencing Core, at Salk Institute.

GRO-seq procedure

For GRO-seq experiments 5x10^6 nuclei were used for run-on reaction. Nuclei isolation, run-on, RNA fragmentation and enzymatic TAP and PNK treatments were performed according to *Wang et al, 2011* with modifications. In brief, 10ml of swelling buffer (10 mM Tris/HCl pH 7.5. 2mM $MgCl_2$, 3mM $CaCl_2$, add fresh 2 U/ml Superase-In) was added to the plates and incubated for 5min on ice. Then, cells were scraped. Pelleted cells were then resuspended with gently shake in 1000 μl of lysis buffer (Swelling buffer with 0.5 % IGEPAL CA-630, 10 % glycerol + 2 U/ml SUPERase-In). After 5min incubation on ice, 9ml of lysis buffer was added and centrifuged at 600x g, 4°C for 5 min. After washing with lysis buffer, 10^7 nuclei were resuspended in 100ul of Freezing buffer (40% glycerol, 5mM MgCl2, 0.1mM EDTA, 50mM Tris pH7.5, 2U/ml SUPERase-In). Then, 50ul of run-on buffer (1.5mM Tris-Cl pH 8.0, 7.5 mM MgCl2, 1.5 mM DTT, 450 mM KCL, 0.6U/μl of SUPERaseIn, 1.5% sarkosyl, 750uM ATP, GTP, and Br-UTP and 4.5 μM CTP) was added. Run-on reactions were incubated during 5' at 30°C. For RNA hydrolysis, samples were treated with RNA fragmentation reagent (Ambion, AM8740) at 70C for 30min. BrU immunoprecipitation was done using anti BrDU beads from Santa Cruz (sc-32323 AC). Decapping, 3' de-phosphorylation and 5' phosphorylation were performed using sequential incubations with 0,5 μl tobacco acid pyrophosphatase (TAP, 5 U, Epicentre), at 37°C for 1.5 h, then 1 μl PNK at 37ºC was added for 30'. Finally, additional 2ul of

PNK along with 20ul ATP 10 mM at 37ºC for 30' was added for 5' phosphorylation. Required RNA purifications throughout the procedure were done using Trizol LS (Life Technologies) and Glycoblue co-precipitant (Life Technologies, AM9516) was added to facilitate RNA precipitation. Before sequencing, the RNA from two independent run-on reactions were pulled and libraries were prepared using NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB#E7300S/L) following manufacturers indications. The amplified barcoded retro-transcribed BrU-RNA was then sent to the Next Generation Sequencing core at Salk Institute and sequenced in Illumina HiSeq 2500 high-throughput sequencing device.

Indirect immunofluorescence

hESCs were fixed with formaldehyde 2% 10min. After permeabilization with Triton 0.1% for 10min, cells were washed with PBS and incubated with blocking solution (PBS Tween 0.1%, BSA 0.1%, FBS 10%) for 30min at room temperature. Primary antibodies diluted in blocking solution were added and incubated overnight at 4°C. After washes, cells were incubated for 2h at room temperature with Alexa-conjugated secondary IgG antibodies. Finally, mounting media containing DAPI was added. Images were captured by Zeiss LSM 780 confocal microscope using ZEN 2011 software.

Coimmunoprecipitation

hESCs were lysed with IPH buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P40, and Protease/Phosphatase Inhibitors (Roche) and incubated 20min on ice. After pelleted the cell debris, pre-cleared cell extracts were incubated with specific antibodies or normal IgGs overnight at 4°C. Then, protein A/G agarose beads (sc-2003) were added to the extract for additional 2h at 4°C. After 4 washes with the IPH buffer, bead conjugates were boiled and loaded into SDS-PAGE gels to analyze the co-immunoprecipitates.

Plasmids, antibodies, probes and primers

Pgl3 basic and promoter vectors from Promega were used as backbones for our luciferases reporter assays. Subsequent cloning of promoter or enhancer sequences was performed using Gibson Assembly Cloning Kit (NEB #E5510). Ligation was done at 50C°C during 15 min. Primers were designed using NEB builder for Gibson Assembly (http://nebuilder.neb.com) and are listed below. All inserts have between 400bp-1000bp and were amplified from genomic DNA by PCR. Other primers, probes as well as antibodies and siRNAs are listed below.

Luciferase Assays

hESCs were plated into single cells using Accutase dissociation reagent. The next day, Pgl3-derived constructs together with pRL-SV40 Renilla control vector were co-transfected using Lipofectamine 3000 reagent (Invitrogen). Following 24h after transfection, hESCs were treated with the indicated cytokines. Then, cells were lysed and processed following Dual-Luciferase® Reporter Assay System Technical Manual (Promega). Luciferase activity was recorded in a 96- well plate luminometer (Thermo Labsystems Luminoskan Ascent). The values of the experimental reporter gene were normalized to Renilla values. The normalized values from three independent biological replicates are shown in the graphs.

ChIP-seq analysis

ChIP fragments were sequenced in an Illumina HiSeq 2500 sequencer. Reads were aligned to the Human hg19 genome assembly (NCBI Build 37) using Bowtie2 (v2.1.0 PMID: 22388286). Only reads that mapped uniquely to the genome were considered for further analysis. Peak finding, motif finding, and peak annotation, genome browser read density files and read density histograms, heatmaps, and metagene profiles were performed using HOMER (v4.6, http://biowhat.ucsd.edu/homer/, PMID: 20513432). Genomic binding peaks for transcription factors were identified using the 'findPeaks' command in HOMER, with default settings of '-style factor': 200-bp peaks, with fourfold tag enrichment and 0.001 FDR significance over background (ChIP input), fourfold enrichment over local tags, and normalization to 10 million mapped tags per experiment. For β-catenin ChIP-seq, only peaks with more 30 reads were considered for the analysis. Histone modification ChIP-Seq parameters were found in similar many using the "-style histone" option, which allows for variable length peaks that may cover large areas. Peaks were assigned to gene targets based on the closest RefSeq defined TSS. Overlapping peaks were defined between peaks with direct overlap by at least on nucleotide. The ChIP-Seq read densities were visualized along the genome using IGV(http://www.broadinstitute.org/igv/, PMID: 22517427). Heatmaps were visualized using Java TreeView (http://jtreeview.sourceforge.net/, PMID: 15180930). De novo motif finding was performed using HOMER using 200 bp sequence fragments centered on ChIP-Seq peak summits. Background sequences were randomly chosen from the genome, matched for GC%.
For the analysis of β-catenin and SMAD peaks distance to the nearest co-regulated gene (Figure S5C and S5D), we selected those peaks encompassing a 100Kb region from the nearest co-regulated TSS. Among this set, those peaks that were closer to a non-coregulated gene than a co-regulated gene were excluded from the analysis.

GRO-seq analysis

GRO-seq libraries were sequenced in an Illumina HiSeq 2500 sequencer. GRO-Seq reads were trimmed to remove 3' A-stretches originating from the library preparation. Each read was then aligned to the human hg19 genome assembly using Bowtie2. Only reads that mapped uniquely to the genome were considered for further analysis. Gene expression levels were calculated using HOMER by summing all + strand reads in gene bodies of RefSeq Genes. Gene counts were then analyzed for differential expression using edgeR. HOMER was used to generate normalized histograms, heatmaps, and genome browser visualization files. To identify Wnt3a and Activin A co-regulated genes, we selected those genes that were induced greater in the co-treatment than in any of the single treatments (FC>2, adj P value< 0.05). The resulting list contains 189 genes (Table S1). Gene ontology analysis of the 189 co-regulated genes was carrying out using DAVID Bioinformatics Resources 6.7 version. To identify those Wnt3a+Activin A genes more induced in YAP depleted cells, we compared the expression values of the 189 co-regulated genes in siYAP (W200A100) and siC (W200A100) conditions. In summary, we found that the nascent transcription of 89 out of the 189 co-regulated genes increased in the siYAP condition (FC>1.25). The list of the 89 YAP-repressed genes is shown in Figure S7A.

List of Antibodies and siRNAs

Antibody	Company	Reference	Specie	Application
β-catenin	Santa Cruz	sc-7199	Rb	ChIP/ChIPseq/WB
LEF-1	Home made	----	Rb	ChIP/ChIPseq/WB
Total RNAPII (N20)	Santa Cruz	sc-899	Rb	ChIP/ChIPseq
Ser5P-RNAPII	Millipore	05-623	Ms	ChIP/ChIPseq
Ser2P-RNAPII	Covance	mms-129r	Ms	ChIP/ChIPseq
Ser7P-RNAPII	Active Motif	61087	Ms	ChIP/ChIPseq
NIPBL	Bethyl	a301-779a	Rb	ChIP/ChIPseq
H3K27ac	Abcam	ab4729	Rb	ChIP/ChIPseq
H3K27me3	Millipore	07-449	Rb	ChIPseq
H3K4me1	Abcam	ab8895	Rb	ChIP
H3	Abcam	ab1791	Rb	ChIP
SMAD2/3	Santa Cruz	sc-8332x	Rb	ChIP
P300	Santa Cruz	sc-584	Rb	ChIP
BRG1	Santa Cruz	sc-17796	Ms	ChIP
RAD21	Abcam	ab992	Rb	ChIP
SMC3	Abcam	ab9263	Rb	ChIP/WB
SMC1	Bethyl	a300-055a	Rb	ChIP/WB
EZH2	Millipore	17-662	Ms	ChIP
CDK9	Santa Cruz	sc-8338	Rb	ChIP/WB
YAP1	Cell Signaling	4912S	Rb	WB
GATA6	R&D	af1700	Goat	IF/WB
SOX1	Cell Signaling	4194S	Rb	WB
Oct3/4	Santa Cruz	sc-9081	Rb	IF/WB
DDX39	Abcam	ab50697	Ms	WB
CRSP70	Santa Cruz	sc-48776	Rb	WB
β-catenin	Santa Cruz	sc-7963	Ms	WB
NELF-A	Santa Cruz	sc-32911	Rb	ChIP
NELF-A	Bethyl	A301-910A	Rb	ChIP
TEAD4	Abcam	ab58310	Ms	ChIP
TAZ	Cell Signaling	4883S	Rb	WB
YAP1	Santa Cruz	sc-101199	Ms	ChIP

siRNA	Company	Reference	Specie	Application
CONTROL	Silencer Select Life Tech.	#1	Human	Transfection
NIPBL	Silencer Select Life Tech.	s24588	Human	Transfection
YAP1	Silencer Select Life Tech.	s20366	Human	Transfection
CDK9	Silencer Select Life Tech.	s2834	Human	Transfection
TAZ	Silencer Select Life Tech.	s24789	Human	Transfection
SMAD2/3	Santa Cruz	sc-37238	Human	Transfection
β-catenin	Santa Cruz	sc-29209	Human	Transfection
MED26	Santa Cruz	sc-38573	Human	Transfection
SMC3	Santa Cruz	sc-38391	Human	Transfection
TEAD4	Santa Cruz	sc-96187	Human	Transfection

List of Primers		
Name and distance to TSS	**Application**	**Fw/Rw**
MIXL1 -12.76 Kb LEF enhancer	ChIP	GCCTTTCATCTGAGCCTTTG
		AGCATTGAAATGGGGAAGTG
MIXL1 -295bp SMAD enhancer	ChIP	GAT TTG ACC CGG AGA AGA GA
		CAT CCC CGA AGG ACT ATT TG
MIXL1 +360bp	ChIP	AGCTGCTGGAGCTCGTCTT
		CCTGAACAGACCTGGAGGAG
MIXL1 +796bp	ChIP	GACACAGATGAGGGGCAGTT
		GCTCAAAGCCAGAGGAAAAC
MIXL1 +2625bp	ChIP	CGTCTCTTCAACCCTCATCC
		GTGAAGATGACAGGCCCAAT
MIXL1 +3.174 bp (close TTS)	ChIP	AATACCTTTTGGGGGCTGTT
		TGGCCTCACTTTGATTTCCT
EOMES -44Kb LEF enhancer	ChIP	GACTTTCTGAATGGGCAGGA
		CCAAGGCTTTGATCTTCCAG
EOMES -9KB LEF enhancer	ChIP	CTGATACCCTGCCCTGTGAT
		GTCCTCCGCTTTGATCTGAC
EOMES -0.1Kb	ChIP	GGGCTGTCACTAGCTGCTTT
		ACCAGCCAATAGGAGGGTCT
EOMES +1.2Kb SMAD enhancer	ChIP	GCTTGTGGACATGCCCTAAT
		CCTGAACAGACCTGGAGGAG
EOMES +1.8Kb	ChIP	TGCCCCAACATCGGTATATT
		AATATGAGCCTTCGGAAGCA
EOMES +4.9Kb (close TTS)	ChIP	GGGGAGGTCGAGGTTCTTAC
		ATGGTAGTCCAGCTGCCATC
NODAL -14Kb enhancer	ChIP	TGCATTCCCCACTAACATCA
		ATTAGCTCTCCCAGGCTTCC
Negative control region	ChIP	GCCTCAGGTGATCCTCTCAC
		GGCATTGTGGCATGTAACTG
MIXL1	cDNA	AGCTGCTGGAGCTCGTCTT
		GCAAGTGGATGTCGGGGTA
EOMES	cDNA	ACTGGTTCCCACTGGATGAG
		ATTTGCGCCTTTGTTATTGG
T	cDNA	ACGCCATGTACTCCTTCCTG
		TGAGCTTGTTGGTGAGCTTG
Gata6	cDNA	AGCGACTCCAGAGCCTTTC
		AGGAGGAGGACGAGGAAGAG
SOX17	cDNA	TTTCATGGTGTGGGCTAAGG
		TTGTGCAGGTCTGGATTCTG
LEF-1	cDNA	TGGAGGCCTCTACAACAAGG
		GATGGGTGGAGAAAGAGATCC

CXCR4	cDNA	GGTGGTCTATGTTGGCGTCT
		CTCACTGACGTTGGCAAAGA
RPS23	cDNA	TGTCGTGGACTTCGTACTGC
		ATGCCACTTCTGGTCTCGTC
LEFTY1	cDNA	AACAGCGAGCTGGTGCAG
		CAGCCACTCGACGGTCAC
ID1	cDNA	CTCCAGCACGTCATCGACTA
		GATTCCGAGTTCAGCTCCAA
FOXA2	cDNA	GACCTCTTCCCCTTCTACCG
		GGCACCTTCAGGAAACAGTC
AXIN2	cDNA	CTTTGTGACCAAGCAGACGA
		GCATGGTGGTGGATGTAGTG
NODAL	cDNA	GAGATTTTCCACCAGCCAAA
		AGGTGACCTGGGACAAAGTG
PAX6	cDNA	GGGCAATCGGTGGTAGTAAA
		TTTCCCAAGCAAAGATGGAC
SOX1	cDNA	CACAACTCGGAGATCAGCAA
		CTCGGACATGACCTTCCACT
POU5F1	cDNA	AGAAGGATGTGGTCCGAGTG
		GCCTCAAAATCCTCTCGTTG
OTX2	cDNA	AAGCACTGTTTGCCAAGACC
		CGGGCAAGTTGATTTTCAGT
EOMES -44Kb enhancer	cloned into pgl3 basic and pgl3 SV40p	gtaaaatcgataaggatccgtcgacAAGAGAAGAGAGGGATACAG
		aaggctctcaagggcatcggtcgaCTTCAAAATCTTATCTAATTGAGACTC
MIXL -12Kb enhancer	cloned into pgl3 basic and pgl3 SV40p	CGGGgtcgacCGTATTGTCACAAGTCCCCTCCTC
		CGGGgtcgacCGTAGCTTTACAGCCTGGAGCAAC
KLF5 -20Kb enhancer	cloned into pgl3 basic	aaatcgataaggatccgtcgacGTTTTAATAGTAAAAACCATATTTGGG
		tctcaagggcatcggtcgacTGTTGTTCTGTGCTTTATAGG
NODAL -25Kb enhnacer	cloned into pgl3 basic	aaatcgataaggatccgtcgacTCTCTGGCTTTTCACAAACCCATG
		tctcaagggcatcggtcgacAGCACCATGCCTGGCACA
MIXL1 promoter	cloned into pgl3 basic	cgggAGATCTcgggTTAGGGAGGCTTGCAGAAGA
		cgggAAGCTTcgtaCGCGGGCCGCACTTATA
MIXL1 SMAD -5Kb from TSS	cloned into pgl3 basic	aaatcgataaggatccgtcgaCCTCTCCACCAAGCCCAT
		tctcaagggcatcggtcgACTCACTGCCTATTCGAAAGAAC
EOMES promoter	cloned into pgl3 basic	ctagcccgggctcgagatcTCCTAACTGTGGGGCTGTTAG
		agcttacttagatcgcagatcTACTGCGCGTACTGGCGC
KLF5 promoter	cloned into pgl3 basic	ctagcccgggctcgagatctAGATACACAGGGACAAGAGC
		agcttacttagatcgcagatcTGGACTCCTCAGACAGCG
NODAL promoter	cloned into pgl3 basic	ctagcccgggctcgagatcTCACCCCGTTGGCTCCATC
		agcttacttagatcgcagatctGCAGCACCTCCAGCCCTT
ID1 promoter	cloned into pgl3 basic	ctagcccgggctcgagatctAACACCAAGCAGATATTAAATATTAGG
		agcttacttagatcgcagatctACAGAATGGGCAAAGCGA

EOMES probe (promoter)	3C	CCTGGGAGGAGGGTG
EOMES Positive control (rev) prom	3C	CACCTTCTTCCAGCGTGTGA
EOMES TSS (anchor)	3C	TTCCTTCCTCGTACCTCTTGCT
EOMES -2.6Kb	3C	GAAAAGCACTGTTTCAGCTACAGATC
EOMES -4Kb	3C	TTGGCCCTTTGTTCTCTGATG
EOMES -6.3Kb	3C	GCGGCATCTGCAAATTAAGG
EOMES -8Kb	3C	CCCACCCCCTCCTTTGTC
EOMES -10Kb	3C	TCTTGTTCTGTTGCCCAAGCT
EOMES -26Kb	3C	ACAGTTCCCTTTTCTTTGGTAGTTGT
EOMES -33Kb	3C	AAGAAGACTCACTTTTCATCGATGGT
EOMES -41Kb	3C	CATGTCTTGGAGAATGTTTCATGTT
EOMES -42Kb	3C	TTTGGATTCCCAATTGAGATCT
EOMES -47Kb	3C	CTGCCCATTTGGGTAGAGATCT
MIXL1 probe (-12Kb, enhancer)	3C	CTCAAATGGTGGTCGCTCAGGCAGT
MIXL1 Positive control (rev) enh	3C	CTCCATGTTAATGGCAAACTCAGT
MIXL1 -12Kb (anchor)	3C	GGGTTCCAGCATTTCCTACAAC
MIXL1 -11Kb	3C	TTGGACGGAGCTGTGTGTGA
MIXL1 -7.6Kb	3C	AACCACAATTCCATTATCTCACATCTA
MIXL1 -2.9Kb	3C	CCAGACTGGGCTGCTGTAGAA
MIXL1 -0.6Kb	3C	GCTGCCCCCTGGATCC
MIXL1 TSS	3C	AGCTGCTGGAGCTCGTCTTC

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