Actin/Smad2 and Wnt/β-catenin up-regulate HAS2 and ALDH3A2 to facilitate mesendoderm differentiation of human embryonic stem cells

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Actin and Wnt signaling are necessary and sufficient for mesendoderm (ME) differentiation of human embryonic stem cells (ESCs). In this study, we report that during ME differentiation induced by Activin and Wnt, Activin/Smad2 induces a decrease of the repressive histone modification of H3K27me3 by promoting the proteasome-dependent degradation of enhancer of zeste 2 polycomb (EZH2)-repressive complex 2 subunit. As a result, recruitment of the forkhead protein FOXH1 on open chromatin regions integrates the signals of Activin/Smad2 and Wnt/β-catenin to activate the expression of the ME genes including HAS2 and ALDH3A2. Consistently, H3K27me3 decrease is enriched on open chromatin around regulatory regions. Furthermore, knockdown of HAS2 or ALDH3A2 greatly attenuates ME differentiation. These findings unveil a pathway from extracellular signals to epigenetic modification-mediated gene activation during ME commitment.

Differentiation of embryonic stem cells (ESCs) into a specific lineage involves the finely tuned coordination between extrinsic signals and intrinsic mediators (1, 2). Among extrinsic factors, TGF-β (transforming growth factor-β) and Wnt signals have been shown to be critical in mesendoderm (ME) specification (3–12). The TGF-β superfamily members Activin/Nodal play a vital role in mesendoderm differentiation during gastrulation (13). Through phosphorylation by the Activin/Nodal receptors, Smad2/3 are activated and then work with Smad4 to regulate gene expression in the nucleus (14–17). The canonical Wnt/β-catenin pathway regulates a variety of cellular events such as cell proliferation, differentiation, and migration and controls embryo patterning (18, 19).

Chromatin accessibility of genes that determine cell fates is modulated by various factors including epigenetic modifiers and chromatin remodelers (2, 11, 13, 20). The inactive or active state of chromatin can be robustly maintained through the cross-talk among different histone modifications. H3K27ac is characterized for open chromatin and actively transcribed genes, whereas trimethylation of histone H3 on lysine 27 (H3K27me3) is a repressive mark linked to silent chromatin and gene repression (21–24). Whole-genome studies have revealed that H3K27me3 occupies critical developmental genes in both human and mouse embryonic stem cells, and often coexists with the active mark H3K4me3, forming a poised state at regulatory regions of differentiation genes (25, 26). Chromatin accessibility depends on the overall effects of those histone modifications upon differentiation of signal stimulation (22). Although open chromatin signals have been reported to be associated with ME, endoderm, and mesoderm differentiation (27), it is unclear how open chromatin is linked to histone modifications and transcription at the early stage of ME differentiation.

Our previous work demonstrated that during ME specification, Activin decreases H3K27me3 levels via the Smad2-mediated reduction of the EZH2 protein, the methyltransferase subunit of polycomb repressive complex 2 (PRC2) (11). To test whether decreased H3K27me3 would result in elevated chromatin accessibility in the early stage of Activin and Wnt-induced ME differentiation, we applied the assay for transposase-accessible chromatin with a high-throughput sequencing (ATAC-seq) approach to assess accessible chromatin. We found that, although Activin stimulation could not alter global open chromatin, the decreased H3K27me3 modification at the transcription start site (TSS) regions elevates chromatin accessibility and thus facilitates the recruitment of β-catenin. Then Smad2 and β-catenin, together with other factors such as FOXH1, activate gene expression to ensure ME specification. From the Activin and Wnt-co-regulated genes, we further found that hyaluronan synthase 2 (HAS2) and aldehyde dehydrogenase 3 family member A2 (ALDH3A2), which are regulated by Smad2–β-catenin–FOXH1, are critical for ME differentiation.

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The ATAC-sequence data for this study were deposited to the Gene Expression Omnibus (GEO) database under GEO accession number GSE113047.

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2 To whom correspondence should be addressed. E-mail: ygchen@tsinghua.edu.cn.
3 The abbreviations used are: ESC, embryonic stem cell; ME, mesendoderm; ALDH3A2, aldehyde dehydrogenase 3 family member A2; ChIP-seq, chromatin immunoprecipitation sequencing; HAS2, hyaluronan synthase 2; PRC2, polycomb repressive complex 2; TGF-β, transforming growth factor-β; RA, retinoic acid; DMEM, Dulbecco’s modified Eagle’s medium; TSS, transcription start site; bFGF, basis fibroblast growth factor; sHNA, short hairpin RNA; qPCR, quantitative PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EZH2, enhancer of zeste 2 polycomb RPKM, reads per kilobase of bin per million.
Results

Smad2 and β-catenin binding motifs are enriched in the open promoter regions of ME markers

We have previously demonstrated that Activin A and Wnt3a could efficiently induce ME differentiation of human ESCs, and Activin A reduced H3K27me3 levels in the early stage of ME commitment (11). As H3K27me3 has been shown to associate with chromatin accessibility (24, 28), we attempted to investigate whether open chromatin was changed during AW-induced ME differentiation. To this end, we performed ATAC-seq with human H1 ESCs upon treatment of 25 ng/ml of Activin A, 25 ng/ml of Wnt3a, or both (AW) for 6 h, but found no significant global open region changes at the early stage of mesendoderm differentiation (Fig. 1A). After analyzing the distribution of those open signals, we found a large percentage of open peaks were enriched around TSS regions (Fig. 1B). In addition, comparing gene expression profiles from the published RNA-seq data (11), we found that although open regions showed little change in the promoters of different lineage genes upon 6 h treatment of AW, the expression of ME genes was up-regulated, whereas no obvious changes in pluripotency, neuroectoderm, or trophoblast markers (Fig. 1C, Table S1), indicating that open chromatin does not ensure gene expression and lineage determination, and other regulatory events should exist to determine ME differentiation.

By analyzing the published ChIP-seq data (Fig. 1D, Table S1) of Smad2, H3K27me3, H3K4me3 (GSE81617) (11), β-catenin, and RNA polymerase II (GSE64758) (10), in combination with our ATAC-seq data (GSE113047), we found that AW-induced Smad2 binding was overlapped with the open chromatin regions around TSS of the ME markers such as EOMES, H9252 (Fig. 1A, Table S1), indicating that open chromatin does not ensure gene expression and lineage determination, and other regulatory events should exist to determine ME differentiation.

As Smad2 has weak DNA binding ability and Smad2 cooperates with chromatin accessibility (24, 28), we investigated whether open chromatin was changed after AW-induced ME differentiation. To this end, we performed ATAC-seq with human H1 ESCs upon treatment of 25 ng/ml of Activin A, 25 ng/ml of Wnt3a, or both (AW) for 6 h, but found no significant global open region changes at the early stage of mesendoderm differentiation (Fig. 1A). After analyzing the distribution of those open signals, we found a large percentage of open peaks were enriched around TSS regions (Fig. 1B). In addition, comparing gene expression profiles from the published RNA-seq data (11), we found that although open regions showed little change in the promoters of different lineage genes upon 6 h treatment of AW, the expression of ME genes was up-regulated, whereas no obvious changes in pluripotency, neuroectoderm, or trophoblast markers (Fig. 1C, Table S1), indicating that open chromatin does not ensure gene expression and lineage determination, and other regulatory events should exist to determine ME differentiation.

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EZH2 degradation is responsible for Activin/Smad2-induced H3K27me3 decrease

Next we explored the relationship of histone modifications and chromatin opening during ME specification at the genome-wide level. As shown in Fig. 2A, there was a close overlap between decreased H3K27me3 modification and open chromatin signals at about ±2.5k of TSS after AW treatment, whereas the H3K4me3 level showed little change.

It has been shown that EZH2 plays a critical role in maintaining H3K27me3 levels during ME differentiation (11, 29). To further confirm the role of Activin/Smad signaling in modulating H3K27me3 levels, we knocked down Smad2 in H1 cells (Fig. 2B), and found that Smad2 depletion did not change the expression of the pluripotency genes OCT4 and NANOG (Fig. 2C). In agreement with our early report (11), AW reduced the levels of H3K27me3 and EZH2, and depletion of Smad2, but not β-catenin, blocked the AW-induced reductions (Fig. 2D). Furthermore, Activin-induced EZH2 ubiquitination was decreased by Smurf2 knockdown (Fig. 2E), and the proteasome inhibitor MG132 attenuated AW-induced EZH2 degradation (Fig. 2F). In line with it, the proteasome inhibitors MG132 and bortezomib, but not the lysosome inhibitor bafilomycin A, blocked Activin-induced EZH2 degradation (Fig. 2G). These data together suggest that Activin/Smad2 signaling promotes EZH2 degradation through the proteasome pathway, reduces H3K27me3 modification, and thus alters the balance of H3K27me3-H3K4me3 in the open chromatin regions.

Smad2 decreases H3K27me3 independent of Smad4

As Smad2 has weak DNA binding ability and Smad2 cooperates with Smad4 to regulate transcription (14–16), we wondered whether the Smad2-mediated H3K27me3 decrease depends on Smad4. Transient depletion of Smad4 did not affect pluripotency gene expression but greatly impaired AW-induced ME differentiation (Fig. 3, A and B). Although knockdown of Smad4 dramatically reduced enrichment of Smad2 on ME genes (Fig. 3C), it had no impact on the Activin/Smad2-induced decrease of H3K27me3 and EZH2 (Fig. 3D). Together these data indicate that Smad4 is dispensable for Smad2-mediated H3K27me3 and EZH2 decrease, but is critical for Smad2 to bind to ME marker genes and contributes to ME differentiation.

Smad2 and β-catenin cooperate with FOXH1 to promote ME differentiation

To explore the possible recruitment of transcription factors to the open chromatin regions, we picked up the top 200 enriched binding motifs of transcription factors in the open chromatin regions upon AW treatment and focused on those involved in lineage determination. Among them, the binding motifs of the mesoderm- or endoderm-related factors (ME: EOMES and T; endoderm: SOX17, FOXA2, and FOXH1) were strongly enriched (Fig. 4A), whereas the ectoderm (PAX6, SOX1, OLIG1) or trophoblast markers (GATA3, CDX2) were not (data not shown). EOMES and T not only serve as ME markers, but also are the critical regulators for ME fate determination (30, 31), suggesting that the open chromatin state provides the accessibility of ME lineage regulators at the early stage of ME differentiation.

As enrichment of the binding motifs for Smad2/Smad3 and T cell factor 4 (TCF4) was also observed, we asked whether knockdown of Smad2 would influence β-catenin binding to the ME gene promoters. As shown in Fig. 4B, the AW-induced occupancy of β-catenin was abolished in Smad2 knockdown cells. As Smad2 mediates the transcription state by promoting EZH2 degradation and reducing H3K27me3 levels, we tested whether the EZH2 inhibitor DZNep could mimic the function of Activin/Smad2 to enhance β-catenin binding to ME gene promoters. Treatment using 25 ng/ml of Wnt3a for 6 h slightly increased β-catenin enrichment on the ME genes T, EOMES, and FGF8, but pretreatment of DZNep for 2 h apparently

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**Figure A**
- Scatter plots showing the relationship between log2(RPKM) values.
- Three panels with different combinations of R values.

**Figure B**
- Bar chart showing the percentage of total peaks.
- Two groups: TSS ±2.5k and Random.

**Figure C**
- Heatmaps for different cell types:
  - Pluripotency
  - Neuroectoderm
  - Trophoblast
- Genes included: DNMT3B, NANOG, SOX2, OCT4, PRDM14, NODAL, WNT3, FGFR8, MIXL1, PAX6, ASCL1, NKX2.1, QLG1, SOX1, GATA3, HAND1, GBX2, GCM1, CDX2.

**Figure D**
- Genome browser tracks for various regions:
  - ATAC
  - H3K27me3
  - H3K4me3
  - Smad2
  - β-catenin
  - FOXH1
  - Pol2
- Chromosome locations and regions highlighted.

**Figure E**
- Bar graphs showing relative occupancy of input (%).
- Genes: Smad2, β-catenin, H3K27me3, H3K27ac, IgG.
- Conditions: Ctrl, AW6.

**Figure F**
- Genome browser tracks for OCT4, SALL4:
  - ATAC
  - H3K27me3
  - H3K4me3
  - Smad2
  - β-catenin
  - FOXH1
  - Pol2
- Chromosome locations.

**Figure G**
- Genome browser tracks for SOX1, CGA:
  - ATAC
  - H3K27me3
  - H3K4me3
  - Smad2
  - β-catenin
  - FOXH1
  - Pol2
- Chromosome locations.
enhanced Wnt3a-induced β-catenin binding (Fig. 4C). These data indicate that Activin/Smad2-altered histone modification is required for β-catenin recruitment to ME genes. Furthermore, knockdown of either Smad2 or β-catenin greatly impaired expression of ME markers (Fig. 4D), indicating that cooperation of Smad2 and β-catenin is critical for AW-induced ME differentiation.

The binding motif of FOXH1 was also enriched by Activin treatment (Fig. 4B). It has been shown that FOXH1 interacts with Smad2 and is required for Activin responses during early embryonic patterning (32–36). Consistently, FOXH1 knockdown dampened ME differentiation (Fig. 4E). By analyzing the FOXH1 ChIP-seq data (GSE29422) (37), we found that its binding pattern overlaps with Smad2 and β-catenin in the regulatory regions of the ME genes, but not in the ectoderm or trophoblast genes (Fig. 1, D, F, and G). Although FOXH1 interacted only with Smad2 but not with TCF4 or β-catenin upon AW treatment (Fig. 4F), depletion of either Smad2 or β-catenin affected FOXH1 binding on ME genes (Fig. 4G), suggesting that both Smad2 and β-catenin are important for FOXH1 recruitment.
HAS2 and ALDH3A2 mediate mesendoderm differentiation

Figure 3. Smad4 is required for AW-induced Smad2 binding to ME gene promoters, but not for H3K27me3 reduction. A, H1 cells transfected with control or Smad4 shRNA were harvested for qPCR, 8, the expression of ME markers was detected by qPCR in Smad4-knockdown H1 cells. C, Smad2 enrichment on the promoters of T, MIXL1, EOMES, and FGFR8 was detected by ChIP-qPCR in Smad4-knockdown H1 cells after treatment with AW for 6 h. D, Smad4-knockdown H1 cells were treated with 25 ng/ml of AW for the indicated times before being harvested for histone extraction and then immunoblotting against H3K27me3.

HAS2 and ALDH3A2 act downstream of Activin and Wnt signaling to promote ME differentiation

To identify the downstream mediators, we picked up 33 genes from our previous RNA-seq data that were significantly up-regulated by Activin and Wnt along mesendoderm differentiation (Fig. 5A, Table S2) (11) and found that HAS2 and ALDH3A2 had strong open chromatin signals with enrichment of FOXH1, Smad2, and β-catenin (Fig. 5B). Although there are three hyaluronan synthase genes, HAS1, HAS2, and HAS3 (38), only HAS2 was co-regulated by AW treatment (Fig. 5C). HAS2 has been shown to function in hESC differentiation to primitive endoderm and mesoderm (39). Aldehyde dehydrogenase 3 family member A2 (ALDH3A2), enriched in skin, adrenal, kidney, and liver, plays a major role in fatty acid metabolism, and its mutations are associated with Sjogren-Larsson syndrome (40). To confirm the importance of Activin and Wnt signaling in the expression of HAS2 and ALDH3A2, we examined their expression in Smad2 or β-catenin-knockdown H1 cells. The expression of HAS2 and ALDH3A2 was induced by AW treatment, but apparently attenuated in the knockdown cells (Fig. 5D). Depletion of FOXH1 had a similar effect. Interestingly, FOXH1 knockdown also significantly impaired the recruitment of Smad2 and β-catenin to the HAS2 and ALDH3A2 genes (Fig. 5E). Taken together, our results indicate that Smad2, β-catenin, and FOXH1 cooperatively regulate the expression of HAS2 and ALDH3A2.

To explore the function of HAS2 and ALDH3A2 in ME specification, we generated knockout H1 cells (Fig. 5F). As shown in Fig. 5G, knockdown of HAS2 strongly attenuated the AW-induced expression of ME markers. ALDH3A2 also had a similar effect albeit to a lesser extent. Aldehyde dehydrogenases are critical in fatty acid metabolism, especially retinoic acid (RA) production (41). To investigate the possible involvement of RA in AW-induced ME specification, we applied different doses of retinoic acid together with Activin A and Wnt3a for 24 h and found that RA had no effect on the AW-induced expression of ME markers in WT H1 cells (Fig. 5H). However, 2 nM RA could rescue the expression of T and MIXL1 in ALDH3A2-knockdown cells (Fig. 5I). Therefore, ALDH3A2 may modulate ME differentiation through its metabolic product RA. These data together indicate the important function of HAS2 and ALDH3A2 in ME specification.

To further confirm the significance of AW-induced target genes in mesoderm and endoderm specification, we conducted a long differentiation assay after AW-induced ME differentiation: 25 ng/ml AW for 96 h for the endoderm lineage and AW for 48 h followed by BMP4 and FGF2 treatment for another 48 h for the mesoderm lineage. Our results revealed that the expression of both mesoderm marker (T) and endoderm markers (FOXA2, SOX17) was attenuated after knockdown of Smad2, β-catenin, HAS2, or ALDH3A2 (Fig. 6A). Furthermore, the impaired expression of mesoderm markers PDGFRα and SOX9 was also observed in knockdown cells (Fig. 6B). Consistent with the mRNA levels, we conducted immunofluorescence experiments and observed that the expression of T, FOXA2, and SOX9 were dampened in knockdown cells (Fig. 6, C–E). Taken
together, these results further demonstrate the important roles of Smad2, β-catenin, HAS2, and ALDH3A2 in the commitment of mesendoderm and the following lineages.

Discussion

Our results highlight an important role of Activin/Smad2 signaling at the early stages of ME differentiation through reducing histone modification H3K27me3 without changing the global open chromatin pattern, increasing accessibility at that region. By cooperating with β-catenin and FOXH1 on accessible chromatin, Smad2 up-regulates the expression of ME marker genes, including HAS2 and ALDH3A2 (Fig. 6F).

H3K27me3 is a marker for chromatin accessibility, and generally a low level of H3K27me3 indicates a more accessible chromatin state for gene transcription (22–24). H3K27me3 levels are the result of balance between the methyltransferase PRC2 and the demethylases JMJD3 and UTX (22). JMJD3 and UTX have been shown to play an important role in endoderm differentiation from human ESCs (42), but our previous data suggest that JMJD3 and UTX may not be involved in H3K27me3 reduction in the early stages of AW-induced ME differentiation (11). Instead, we found that Activin reduces the H3K27me3 levels and the PRC2 methyltransferase EZH2 with a similar pattern, and their decrease depends on Smad2, but not Smad4 or Wnt/β-catenin. Furthermore, Activin/Smad2 promotes EZH2 polyubiquitination and degradation via the proteasome pathway. It has been reported that EZH2 down-regulation enhances neuron differentiation of human mesenchymal...
stem cells and Smurf2 is responsible for the EZH2 decrease (43). EZH2 knockout led to H3K27me3 decrease and reduced self-renewal and proliferation of human embryonic stem cells and their full differentiation into mature specialized tissues (29), indicating the complex role of EZH2 in cell lineage commitment.

FOXH1 was identified as a Smad2 DNA-binding partner to mediate Activin signaling during early embryo patterning (33, 34, 44). Foxh1 null mice show defects in development of the anterior primitive streak, axial patterning, and endoderm formation (36). FOXH1 is also involved in endoderm specification of human ESCs (37). It has been demonstrated that Activin signaling disrupts the repression of FOXH1 by the NuRD corepressor complex, resulting in the cooperative action of FOXH1 and Smad2 to activate ME genes (45). Our data indicate that AW treatment enhances FOXH1 binding to ME gene promoters, and this recruitment needs both Smad2 and β-catenin. In agreement with this, knockdown of β-catenin greatly reduces FOXH1 promotion of cell proliferation and invasion (46).

Our data showed that the hyaluronan synthase HAS2 is induced by AW treatment and mediates ME specification, which is consistent with the demonstrated role of HAS2 in the formation of endoderm (39, 47). The aldehyde dehydrogenases are critical in fatty acid metabolism, especially the retinoic acid production (41), and retinoic acid can cooperate with various signals to promote lineage specification and tissue development (48, 49). Our data also suggest that ALDH3A2-mediated retinoic acid generation is important for AW-induced ME specification. Therefore, our work indicates that Activin/Smad2 signaling controls the epigenetic modifications and by cooperating with Wnt/β-catenin signaling, regulates the expression of ME-specifying genes.

**Experimental procedures**

**Cell culture and differentiation of human ESCs**

H1 human embryonic stem cells (WiCell, 12-W0296) were cultured on feeder cells with DMEM/F-12 (Gibco) supplemented with 20% knockout serum replacement (Gibco), 1 mM...
L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 10 ng/ml of recombinant human bFGF. Although for feeder-free culture, H1 cells were maintained on a Matrigel (BD Biosciences)-coated plate with N2B27 medium (DMEM/F-12 supplemented with N2, B27, 1 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 20 ng/ml of recombinant human bFGF) (Table S3). For mesendoderm differentiation, H1 cells were maintained on a Matrigel-coated plate with N2B27/bFGF medium to about 50–60% confluence, and then differentiation was conducted in B27 medium (DMEM/F-12, B27, 1 mM L-glutamine, 0.1 mM nonessential amino acids) supplemented with Activin A (25 ng/ml each) and for the first 48 h and then replaced with 20 ng/ml of BMP4 and 40 ng/ml of FGF2 for another 48 h and harvested by qPCR to assess the expression of the mesoderm genes PDGFRA and SOX9. The data are shown as mean ± S.E. (n = 9, including 3 biological replicates and 3 technical replicates). C, H1 cells expressing control shRNA or Smad2, β-catenin, HAS2, or ALDH3A2 shRNA were cultured on Matrigel-coated plates and treated with 25 ng/ml of Activin A and 25 ng/ml of Wnt3α for 48 h. Anti-T immunofluorescence (1:100) was conducted. D, H1 cells were treated with 25 ng/ml of Activin A and 25 ng/ml of Wnt3α for 48 h, then replaced with 20 ng/ml of BMP4 and 40 ng/ml of FGF2 for another 48 h. Anti-SOX9 immunofluorescence (1:100) was conducted. E, H1 cells were treated with 25 ng/ml of Activin A and 25 ng/ml of Wnt3α for 48 h, then replaced with 20 ng/ml of BMP4 and 40 ng/ml of FGF2 for another 48 h. Anti-SOX9 immunofluorescence (1:100) was conducted. F, schematic model. In the ES stage, the regulatory regions of lineage markers maintain a poised state with both H3K27me3 and H3K4me3 modifications. Upon AW treatment, Smad2 reduces H3K27me3 through EZH2 degradation, and then with the recruitment of β-catenin and FOXH1, together they cooperate to activate transcription of ME genes.

shRNA-mediated knockdown

All shRNA plasmids were obtained from the Sigma TRC shRNA library (Table S4), and a nontargeting nucleotide plasmid served as control. shRNAs were packed into lentivirus following the instruction. H1 cells were cultured in a feeder-free condition and infected with shRNA lentiviral supernatant. Then puromycin (1 μg/ml; Invitrogen) was added into culture medium for 3–5 days to select stable knockdown cells.

Histone extraction

H1 cells were lysed with TEB buffer (PBS containing 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 0.02%
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$\text{Na}_2\text{S}_2\text{O}_3$ added to the proteinase inhibitor mixture (Roche Applied Science) at 4°C for 15 min and then centrifuged at 2000 rpm for 10 min. After removing the supernatant and washing the pellets twice with 500 µl of TEB buffer with proteinase inhibitors at 4°C, the pellets were suspended with 0.2 N HCl and incubated overnight at 4°C. Then histones were precipitated with 100% TCA at a final concentration of 33% and incubated for 1–2 h on ice. After centrifugation at 4°C for 10 min, the supernatant was discarded, and the histone pellets were washed with ice-cold acetone, the pellets were air-dried for 5 min and dissolved with a suitable volume of 150 mm NaCl.

**ATAC-seq library preparation, sequencing, and data processing**

The ATAC-seq libraries of hESCs were prepared as previously described with minor modifications (24). Briefly, samples were lysed in lysis buffer (10 mm Tris-HCl (pH 7.4), 10 mm NaCl, 3 mM MgCl$_2$, and 0.5% Nonidet P-40) for 10 min on ice. Immediately after lysis, cells were then incubated with the Trn5 transposome and tagmentation buffer at 37°C for 30 min (Vazyme Biotech TD502), and the stop buffer was added directly into the reaction to end the tagmentation. PCR was performed to amplify the library for 12 cycles using the following PCR conditions: 72°C for 3 min; 98°C for 30 s; and thermostating at 98°C for 15 s, 60°C for 30 s and 72°C for 3 min; followed by 72°C for 5 min. After the PCR, libraries were purified with the 1.5× AMPure (Beckman) beads. The ATAC-seq library was subjected to sequencing on HiSeq2500 or Xten (Illumina) according to the manufacturer’s instruction. All ATAC-seq reads were first aligned to hg19 using Bowtie2 (version mina) according to the manufacturer’s instruction. All ATAC-seq reads were first aligned to hg19 using Bowtie2 (version 2.2.1) into an FPKM matrix using default parameters, and the normalized with Cuffnorm from the Cufflinks package (version 2.2.2). The paired-end ATAC-seq reads were aligned with the parameters: -t -q -N1 -L25 -X2000 no-mixed no-discordant. All unmapped reads, non-uniquely mapped reads, and PCR duplicates were removed. For downstream analysis, we normalized the read counts by computing the numbers of reads per kilobase of bin per million of reads (RPKM). To visualize the ATAC-seq signal in the UCSC genome browser, we extended each read by 250 bp and counted the coverage for each base. All ATAC-seq peaks were called by MACS (version 1.4.2) with the parameters -nolambda -nomodel. Motif analysis was conducted by HOMER (hypergeometric optimization of motif enrichment) following the instructions.

**RNA-seq data processing**

Paired end reads were first trimmed of the first 15 bp from each end and then mapped to the human genome (hg19) using STAR (version 2.4.0d). Gene expression was estimated and normalized with Cuffnorm from the Cufflinks package (version 2.2.1) into an FPKM matrix using default parameters, and the expression matrix was log$_2$-transformed.

**Quantitative RT-PCR, ChIP, immunoprecipitation, immunoblotting, and immunofluorescence**

These were performed as previously described (11). The primers for qPCR and ChIP-qPCR are listed in Tables S5 and S6. Antibodies used in this study are summarized in Table S3.

**Statistical analysis**

All of the values are shown as mean ± S.E. with a two-way analysis of variance test. The significance between groups was determined by Student’s t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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