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Inhibiting Smad2/3 Signaling In Pluripotent Mouse Embryonic Stem Cells Enhances Endoderm Formation By Increasing Transcriptional Priming Of Lineage-Specifying Target Genes

Øyvind Dahle and Michael R. Kuehn*

Basic Research Laboratory, National Cancer Institute, National Institutes of Health, Frederick, Maryland

Abstract

Background—Pluripotent embryonic stem cells (ESCs) offer great potential for regenerative medicine. However, efficient *in vitro* generation of specific desired cell types is still a challenge. We previously established that Smad2/3 signaling, essential for endoderm formation, regulates target gene expression by counteracting epigenetic repression mediated by Polycomb Repressive Complex 2 (PRC2). Although this mechanism has been demonstrated during differentiation and reprogramming, little is known of its role in pluripotent cells.

Results—Chromatin immunoprecipitation-deep sequencing of undifferentiated mouse ESCs inhibited for Smad2/3 signaling identified *Prdm14*, important for protecting pluripotency, as a target gene. Although *Prdm14* accumulates the normally repressive PRC2 deposited histone modification H3K27me3 under these conditions, surprisingly expression increases. Analysis indicates that increased H3K27me3 leads to increased binding of PRC2 accessory component Jarid2 and recruitment of RNA polymerase II. Similar increases were found at the Nodal endoderm target gene Eomesodermin, but it remained unexpressed in pluripotent cells as normal. Upon differentiation, however, Eomesodermin expression was significantly higher than in cells that had not been inhibited for signaling prior to differentiation. In addition, endoderm formation was markedly increased.

Conclusions—Blocking Smad2/3 signaling in pluripotent stem cells results in epigenetic changes that enhance the capacity for endoderm differentiation.

Keywords

Smad2/3; Nodal signaling; embryonic stem cells; pluripotency; transcriptional priming; *Prdm14*; Jarid2; Polycomb Repressive Complex 2

INTRODUCTION

Pluripotent embryonic stem cells (ESCs) derived from the early embryo offer great potential for regenerative medicine. Realizing this potential will require an understanding of how the balance between pluripotency and cell fate commitment is regulated, a process achieved *in*

*Correspondence to: Michael R. Kuehn, NCI-Frederick, Frederick, MD 21702. mkuehn@mail.nih.gov.

vivo by interaction between intercellular signaling pathways and the intrinsic regulatory networks established by each cell's unique developmental history (Daley 2015). Elucidation of these interdependent processes is critical to achieving differentiation protocols that will allow specific differentiated cell types to be efficiently generated *in vitro*.

Exactly how pluripotent cells become responsive to developmental signals remains ill defined but requires disrupting the network of extrinsic and intrinsic factors that maintains naïve pluripotency (Hackett & Surani 2014). A key feature of pluripotent mouse ESCs is that they are shielded from the pro-differentiation effects of autocrine Fibroblast growth factor (FGF) signaling (Lanner & Rossant 2010). Recently, the transcription factor *Prdm14* was shown to protect pluripotency by, in part, suppressing FGF signaling (Chan et al. 2013; Yamaji et al. 2013). *Prdm14*, in concert with *Jarid2*, was found to promote Polycomb repressive complex 2 (PRC2)-mediated repression of genes encoding components of the FGF pathway. PRC2 catalyzes trimethylation of lysine 27 of histone H3 (H3K27me3), which is recognized by other chromatin modifying complexes that mediate additional events leading to transcriptional silencing (Conway et al. 2015). *Jarid2* was the first identified member of the Jumonji C domain-containing family. Other members act as H3K27me3 demethylases, but *Jarid2* lacks this activity (Landeira & Fisher 2011). Instead, *Jarid2* is a PRC2 component specifically found in pluripotent cells and downregulated upon differentiation, which can recruit the paused form of RNA polymerase II (RNAPII) to lineage-specifying loci that are inactive until cells begin to differentiate (Peng et al. 2009; Landeira et al. 2010; Pasini et al. 2010). This transcriptional priming is thought to allow rapid expression in response to appropriate developmental cues, with PRC2 poised to repress these genes in inappropriate lineages. An unexplored aspect of this mechanism is whether the relevant developmental signals have any influence on the priming process in pluripotent cells.

A key signaling pathway for early cell fate decisions is initiated by the extracellular TGF-beta like factors Nodal and Activin, which activate the downstream nuclear effectors *Smad2* and *Smad3* (*Smad2/3*). We have recently defined a novel paradigm for this pathway: activated *Smad2/3* recruits the Jumonji C domain-containing histone demethylase *Jmjd3* to remove the repressive H3K27me3 modification deposited by PRC2, thereby allowing target gene expression. We have found this mechanism in the differentiation of mouse ESCs and in reprogramming of mouse fibroblasts (Dahle et al. 2010; Dahle & Kuehn 2013), while it was reported independently in the differentiation of human ESCs (hESCs) into endoderm (Kim et al. 2011). In undifferentiated ESCs, blocking *Smad2/3* signaling leads to an increase in H3K27me3 levels at the *Oct4* locus, encoding one of the key pluripotency transcription factors. However, a decrease in *Oct4* gene expression is only found once cells begin to differentiate, suggesting that the full elaboration of this signaling paradigm occurs only after cells exit the pluripotent state (Dahle & Kuehn 2013). This lack of effect on target gene expression prior to differentiation could be related to distinct regulatory functions for PRC2 in pluripotent cells (Aloia et al. 2013).

Here we have carried out further investigation of this paradigm, identifying *Prdm14* as a novel *Smad2/3* target in undifferentiated ESCs. *Prdm14* was recently shown to be negatively regulated by *Smad2/3* signaling in epiblast stem cells (EpiSCs) and in hESCs, both

considered in a primed pluripotent state, rather than naïve (Martello & Smith 2014). We find *Prdm14* is also negatively regulated by Smad2/3 in undifferentiated ESCs, suggesting signaling may also indirectly promote differentiation by contributing to destabilization of the pluripotent state. *Prdm14* gains H3K27me3 with Smad2/3 inhibition, yet surprisingly is not repressed. We find this increase in H3K27me3 leads to enrichment of Jarid2 and RNAPII, providing a mechanistic basis for increased expression and providing an example of a non-repressive role for PRC2 in pluripotent cells. Similar enrichment of Jarid2 and RNAPII was found at genes that drive endoderm differentiation. Intriguingly, while expression of these genes remained low in undifferentiated ESCs they were induced to significantly higher levels upon differentiation in the absence of further Smad2/3 signaling inhibition. The propensity to differentiate into endoderm was also markedly enhanced. These findings suggest that Smad2/3 signaling in pluripotent ESCs normally modulates Jarid2-PRC2 dependent priming of endoderm-specifying target genes, thereby allowing balanced differentiation potential. Inhibition of this pathway specifically in pluripotent cells, therefore, provides a novel strategy for increasing differentiation along the endoderm lineage.

RESULTS AND DISCUSSION

Limited number of loci accumulate H3K27me3 in pluripotent ESCs blocked for Smad2/3 signaling

To further explore the role of the Smad2/3 pathway in counteracting PRC2 function in pluripotency, we chemically inhibited signaling and then mapped genome-wide changes in H3K27me3 using chromatin immunoprecipitation with antibodies against H3K27me3 and deep sequencing (ChIP-seq). Prior to ChIP-seq analysis, undifferentiated ESCs were either left untreated or treated for 96 hours with the small molecule SB431542, which is a specific inhibitor of the Type I Activin Like Kinase receptors for Nodal and Activin signaling. We also carried out ChIP-seq to assess changes in H3K27me3 levels occurring upon differentiation. ESCs were induced to differentiate by removing LIF, and either left untreated or treated with SB431542 (hereafter abbreviated ALKi for Activin Like Kinase inhibitor) for 96 hours. The results from these two analyses showed that blocking Smad2/3 activity in pluripotent stem cells results in a much more restricted increase in H3K27me3 levels than does inhibiting the pathway during differentiation (Fig. 1A, left panel). Only 222 genomic sites unique to pluripotent stem cells showed more than a two-fold increase in H3K27me3 levels upon Smad2/3 activity inhibition (Fig. 1A, left panel, blue ellipse). In contrast, 1332 sites showed increased H3K27me3 specifically during differentiation (Fig. 1A, left panel, pink ellipse). There were also 193 sites that showed increased H3K27me3 under either condition (Fig. 1A, left panel, intersect of ellipses). Although not the goal of this study, it was interesting to note that a total of 1398 genomic sites showed reduced H3K27me3 levels in pluripotent stem cells upon inhibiting Smad2/3 activity (Fig. 1A, right panel, blue ellipse). This is approximately six fold more than the number with increased H3K27me3. Conversely, only 371 sites showed reduced H3K27me3 levels when signaling was inhibited during differentiation (Fig. 1A, right panel, pink ellipse), approximately three fold less than the number of sites with increased H3K27me3. Thus, blocking Smad2/3 activity in these two cellular contexts results in opposite effects on H3K27me3 levels.

Smad2/3 signaling negatively regulates *Prdm14* despite reducing H3K27me3 levels

Further analysis of the ChIP-seq results from pluripotent stem cells treated with ALKi revealed only 64 distinct gene regions with more than a three-fold increase in H3K27me3. Many of these were found to encode proteins involved in developmental signaling that might be relevant for subsequent differentiation. Several are in the FGF signaling pathway, essential for exit from self-renewal and the initiation of differentiation (Lanner & Rossant 2010), suggesting cross talk with Smad2/3 signaling is critical to this function. *Prdm14*, which protects naïve pluripotency by promoting Polycomb repression of FGF pathway components, was also identified. *Prdm14* was recently shown to be regulated by Smad2/3 in hESCs and EpiSCs (Sakaki-Yumoto et al. 2013). Together, these data suggest that normal Smad2/3 activity may have a previously unknown role in regulating pluripotency and the capacity of stem cells to differentiate, by counteracting Polycomb at the *Prdm14* locus.

To investigate this potential role, we first verified that H3K27me3 becomes enriched at *Prdm14* upon inhibition of Smad2/3 signaling. Using ChIP, we found a greater than 3 fold increase in H3K27me3 levels at the *Prdm14* promoter following 96 hours of ALKi treatment (Fig. 1B, $p=0.04$). We next checked expression, expecting either no change as we previously found for *Oct4* (Dahle & Kuehn 2013), or a decrease given that Polycomb deposition of H3K27me3 is normally associated with repression. Surprisingly, we found *Prdm14* expression increased approximately 2.5 fold with ALKi treatment (Fig. 1C, blue bars, $p=0.001$). PRC2 deficient *Suz12*^{-/-} ESCs were found to have very low *Prdm14* levels, again suggesting Polycomb dependent expression (Fig. 1C, green bars). Thus, although normally Smad2/3 activity limits PRC2 mediated deposition of H3K27me3 at *Prdm14* in undifferentiated stem cells, presumably through recruitment of H3K27me3 demethylases to the *Prdm14* promoter, this does not relieve Polycomb repression. Rather, the above results suggest that PRC2 function is important for maintaining active *Prdm14* transcription in pluripotent stem cells, and Smad2/3 signaling counteracts this function thereby reducing expression.

Smad2/3 signaling limits Jarid2-PRC2 and RNAPII accumulation at *Prdm14*

How might active *Prdm14* transcription be dependent on Polycomb, rather than repressed by it? Recent work has shown that Polycomb complexes containing Jarid2, which are specifically found in pluripotent stem cells, can enhance the transcriptional potential of inactive lineage specific genes by recruiting paused RNAPII (Landeira & Fisher 2011). Jarid2-PRC2 complexes might therefore play a similar role of RNAPII recruitment, in this case to a gene already active in stem cells. Based on this hypothesis, we would expect to find higher levels of Jarid2 and RNAPII at *Prdm14*. Indeed, using ChIP we found a three-fold increase in Jarid2 binding at the *Prdm14* promoter upon inhibition of Smad2/3 activity (Fig. 1D, blue bars, $p<0.001$). In keeping with a required role for PRC2, ALKi treatment of *Suz12*^{-/-} ESCs did not result in any significant change in Jarid2 binding at the *Prdm14* promoter (Fig. 1D, green bars, $p=0.25$). We also found a significant increase in binding of RNAPII including the hyper-phosphorylated, elongating form in ALKi treated wild type ESCs (Fig. 1E, blue bars, $p<0.001$). This is also PRC2 dependent, as inhibiting Smad2/3 activity in *Suz12*^{-/-} ESCs did not cause any increase (Fig. 1E, green bars). These results suggest that normally in pluripotent stem cells, Jarid2 containing Polycomb complexes

recruit RNAPII to the *Prdm14* promoter to augment ongoing transcription. Smad2/3 pathway dependent H3K27me3 demethylase activity counteracts this Jarid2-PRC2 dependent process, thereby modulating transcriptional output of this key pluripotency gene and indirectly promoting differentiation.

Blocking Smad2/3 signaling in pluripotent cells enhances subsequent endoderm formation

It was recently shown that *Prdm14* overexpression prevents spontaneous differentiation of mouse ESCs cultured in LIF and serum, and also abrogates differentiation induced by LIF removal (Grabole et al. 2013). Although the degree of *Prdm14* overexpression in that study was not specified, it is likely to be far higher than that induced by blocking Smad2/3 signaling. Indeed, we had not previously noticed any change in the ability of ALKi treated ESCs to differentiate (Dahle & Kuehn 2013). However, to confirm this under the specific conditions used here, and to examine the capacity for ESCs treated with ALKi in the pluripotent state to undergo endodermal differentiation, we removed LIF and discontinued ALKi treatment at the same time. After 96 hours, we carried out immunostaining for the pluripotency marker Oct4 and the endoderm specific marker Eomes, and also used Hoechst dye to stain nuclei (Fig. 2A-D). In this analysis, undifferentiated pluripotent cells positive for Oct4 and negative for Eomes are labeled in green, whereas endoderm cells are negative for Oct4 and positive for Eomes and labeled red. Cells that express both Oct4 and Eomes are labeled yellow, and may represent primitive streak-like cells that will go on to differentiate into endoderm (Trott & Martinez Arias 2013). In control experiments, cells grown continuously in the presence of LIF, either with or without ALKi, showed no Eomes staining and were predominantly Oct4 positive (Fig. 2A, B). 96 hours following LIF removal, immunostaining revealed a mixture of undifferentiated and differentiated cells (Fig. 2C, D). Thus, pre-treatment with ALKi does not elevate *Prdm14* to such a degree that differentiation is prevented.

Interestingly, quantification of the different differentiated cell types, done by comparing to the total cell count determined by Hoechst staining, revealed striking changes for the ALKi pretreated population (Fig. 2E). In control cells that had not been pre-treated with ALKi, 10% were Eomes⁺ Oct4⁺ endoderm-committed cells (Fig. 2E, top yellow bar), whereas approximately 3% were Eomes⁺ Oct4⁻ endoderm (Fig. 2E, top red bar). In the ALKi pre-treated population, there was no significant change in endoderm-committed cells (Fig. 2E, bottom yellow bar, p=0.18), however there was more than a 5-fold increase in endoderm (Fig. 2E, bottom red bar, p=0.04). Thus, although inhibiting Smad2/3 signaling concomitant with LIF removal blocks endoderm formation, pre-treatment with ALKi has the opposite effect, leading to a significant increase.

Enhanced transcription of endoderm-specifying genes during differentiation of ALKi treated pluripotent cells

To investigate how inhibiting Smad2/3 signaling in pluripotent cells enhances endoderm differentiation, we examined the expression of *Eomes*, *Foxa2* and *Sox17*, which encode transcription factors regulating progressive steps in formation of this lineage. We also looked at *Nodal* and *Oct4*, two genes we have previously characterized as Smad2/3 targets whose expression is abrogated in cells differentiating in ALKi but unchanged in ALKi treated

pluripotent cells. We also examined expression of *Nestin* as a negative control. As before, ESCs that had either been cultured normally or treated with ALKi for 96 hours were induced to differentiate by removing LIF, with ALKi treatment stopped at that time. Gene expression was then analyzed 24, 48 and 96 hours after LIF (and ALKi) removal using quantitative reverse transcriptase PCR (Fig. 3). As we previously found, *Oct4* and *Nodal* levels in undifferentiated cells were unchanged by ALKi treatment (Fig. 3A, B left panels, zero time point). However, while untreated control cells also showed no change in *Oct4* levels over 96 hours culture without LIF (Fig. 3A, left panel, light blue line, $p=0.33$), ALKi pre-treated cells showed a greater than 3-fold increase over this time period (Fig. 3A, left panel, dark blue line, $p=0.03$). Interestingly, there was less than a 1-fold increase in similarly treated *Suz12*^{-/-} ESCs (Fig. 3A, right panel, dark green line, $p=0.06$), suggesting some degree of Polycomb dependency. For *Nodal*, expression in untreated control cells showed a small decrease over 96 hours culture without LIF (Fig. 3B, left panel, light blue line, $p=0.005$). However, in ALKi pre-treated cells there was almost a 3-fold up-regulation (Fig. 3B, left panel, dark blue line, $p=0.01$). In *Suz12*^{-/-} ESCs, initial *Nodal* levels were somewhat higher in ALKi pretreated cells, but levels did not increase significantly over 96 hours without LIF (Fig. 3B, right panel, $p=0.18$). Thus the increase in *Nodal* is also Polycomb dependent and likely contributes to the enhanced endoderm differentiation, by providing additional Smad2/3 signaling through a positive feed-forward mechanism. The increase in *Oct4* expression may also be contributing; recent findings have shown that high levels of Oct4 can specify mesendoderm in hESCs under certain conditions (Wang et al. 2012). For *Eomes*, which acts early in endoderm differentiation, expression in wild type ESCs increased approximately 1-fold over 96 hours of normal differentiation (Fig. 3C, left panel, light blue line, $p=0.04$). However, pre-treatment with ALKi led to greater than 3-fold higher expression levels (Fig. 3C, left panel, dark blue line, $p=0.005$). In untreated *Suz12*^{-/-} ESCs there was no increase in *Eomes* expression over 96 hours without LIF (Fig. 3C, right panel, light green line, $p=0.004$), and pretreatment with ALKi led to only a 1.5 fold increase (Fig. 3C, right panel, dark green line, $p<0.001$), again indicating dependence on PRC2 function. Analysis of *Foxa2*, which acts later in endoderm differentiation, showed an increase in expression only in ALKi pre-treated wild type ESCs (Fig. 3D, left panel, dark blue line, $p=0.02$). In *Suz12*^{-/-} ESCs, *Foxa2* expression was higher regardless of treatment. In ALKi treated undifferentiated cells, *Sox17* expression was already at an elevated level (Fig. 3E, left panel, dark blue line, time point zero), and there was no further increase with differentiation. In *Suz12*^{-/-} ESCs, initial *Sox17* expression was elevated regardless of treatment (Fig. 3E, right panel, time point zero). These latter results suggest that PRC2-mediated repression is required to maintain *Foxa2* and *Sox17* expression at low levels in pluripotent cells. As expected, ALKi pre-treatment had no significant effect on expression of the endoderm independent gene *Nestin* in either wild type (Fig. 3F, left panel, $p=0.3$) or in *Suz12*^{-/-} ESCs (Fig. 3F, right panel, $p=0.1$). Thus, blocking Smad2/3 signaling in undifferentiated wild type ESCs significantly enhances expression of these endoderm-specifying genes during subsequent differentiation. That this effect is either lessened or not observed in *Suz12*^{-/-} ESCs for *Oct4*, *Nodal* and *Eomes* suggests that PRC2 dependent transcriptional priming is occurring at these loci in pluripotent stem cells.

Smad2/3 signaling in pluripotent cells limits Jarid2-PRC2 and RNAPII accumulation at endoderm-specifying loci

To provide further evidence for transcriptional priming, we asked if inhibition of Smad2/3 signaling in undifferentiated ESCs leads to enrichment of H3K27me3, Jarid2 and RNAPII at *Oct4*, *Nodal* and *Eomes*. We also analyzed *Foxa2* and *Nestin* again as a negative control. Indeed, ChIP analysis for H3K27me3 showed significantly higher levels at the *Oct4* and *Nodal* loci in ALKi treated compared to untreated ESCs (Figs. 4A & B, left panels, $p=0.02$). For *Eomes*, H3K27me3 levels were already high in untreated ESCs and ALKi treatment induced only a 2-fold increase (Figs. 4C, left panel, $p=0.1$). Similarly, *Foxa2*, which is also inactive in ESCs, exhibited high H3K27me3 levels in untreated ESCs that were increased only 1.5-fold upon ALKi treatment (Fig. 4D, left panel, $p=0.08$). However, for *Eomes* a significant increase in binding of Jarid2 and RNAPII was found (Fig. 4C, middle and right panels, blue bars, $p=0.05$), suggesting a change in composition of the associated Polycomb complexes. There was also significantly increased binding of Jarid2 and RNAPII at the *Nodal* locus (Fig. 4B, middle and right panels, blue bars, $p=0.008$), and a significant increase in RNAPII at *Oct4* (Fig. 4A, right panel, blue bars, $p=0.01$). In ALKi treated *Suz12*^{-/-} ESCs, there was no significant change in Jarid2 or RNAPII binding at *Oct4*, *Nodal* and *Eomes* (Fig. 4A-C; middle and right panels, green bars, $p=0.1$), again supporting PRC2 dependency. For *Foxa2*, there was no significant enrichment in Jarid2 and RNAPII in either wild type or *Suz12*^{-/-} ESCs (Fig. 4D, middle and right panels, $p=0.15$). Thus, the increased expression seen for *Foxa2* is likely an indirect effect of the increase in *Nodal* expression and resulting increase in Smad2/3 recruitment of Jmjd3 to counteract Polycomb. *Eomes*, which has also been shown to interact with Jmjd3 (Kartikasari et al. 2013), may contribute to this same mechanism. The negative control *Nestin* did not show any significant changes in H3K27me3 levels or in Jarid2 and RNAPII binding upon ALKi treatment in either wild type or *Suz12*^{-/-} ESCs (Fig. 4E, $p=0.14$). Taken together, these findings suggest that inhibiting Smad2/3 signaling in pluripotent ESCs leads to increased transcriptional priming of the earliest endoderm-specifying target genes in a process dependent on Jarid2 containing Polycomb complexes.

Controlling RNAPII activity is the ultimate outcome of the extensive network of signaling pathways, epigenetic regulators and transcription factors that control cell fate decisions during development. Indeed, the functional status of RNAPII at gene promoters, whether in the paused form or competent for elongation, has been proposed to be a critical developmental checkpoint (Levine 2011). Our study demonstrates how Smad2/3 signaling can regulate this checkpoint and influence cell fate decisions by counteracting the recruitment of RNAPII carried out by PRC2-Jarid2 complexes. We propose two fundamental activities for Smad2/3 signaling in pluripotent cells. First, it contributes to destabilization of pluripotency by antagonizing PRC2-Jarid2 dependent augmentation of *Prdm14* expression (Fig. 5A). Second, it counters the PRC2-Jarid2 dependent priming of the same target genes subsequently activated by this signaling pathway during endoderm formation (Fig. 5B). This provides an elegant negative feed-forward mechanism to modulate the formation of endoderm, thereby allowing a balanced, and appropriate differentiated cellular make-up. Manipulating this pluripotent stem cell specific “transistor” using small molecule inhibition provides a novel strategy for increasing the efficiency and specificity of *in vitro*

differentiation, a necessary step for translating stem cell technology into clinical applications.

Experimental Procedures

Cell Culture

Wild type E14tg2a ESCs were obtained from BayGenomics. *Suz12* gene trap ESCs were a generous gift from Dr. K. Helin (University of Copenhagen). ES cells were maintained feeder-free and grown in DMEM-KO medium (Invitrogen) supplemented with 10% FBS (Invitrogen), LIF (Millipore), Glutamax (Invitrogen) and Non-Essential Amino Acids (Invitrogen). For Smad2/3 signaling inhibition, cells were treated with SB431542 (Sigma) at 10 μ M for 96 hours. Differentiation was initiated by removing LIF.

Antibodies

Anti-H3K27me3 was from Abcam. Normal rabbit IgG and normal mouse monoclonal IgG were from Millipore. Anti-Oct4 (N19) goat polyclonal antibodies were from Santa Cruz. Anti-Smad2/3 was from BD biosciences. Anti-RNA polymerase II CTD-repeat (ab5131), anti-Eomes (ab23345), and anti-Jarid2 (ab178561) antibodies were from Abcam. Secondary Alexa-488 and Alexa-633 conjugated anti-light chain antibodies were from Jackson ImmunoResearch.

Immunofluorescence staining

ESCs were grown on cover slips in 6-well plates, and then fixed in 4% PFA for 15 min at room temperature. Cells were washed three times for 5 minutes each in 0.1% Tween 20 in PBS, then blocked with 3% BSA, 0.1% Tween 20 in PBS for 30 minutes. Subsequently, cells were incubated with primary antibodies diluted in 3% BSA, 0.1% Tween 20 in PBS for 1 hour, washed as above, and further incubated with either Alexa-488 or Alexa-633 conjugated secondary antibodies diluted in PBS. Primary antibodies were used at 1:500 dilution, and secondary antibodies were used at 1:2000. After three washes in 0.1% Tween 20 in PBS, coverslips were mounted using glycerol containing Hoechst (for DNA staining) before acquiring images.

Gene expression analysis

cDNA was generated with Superscript III (Invitrogen) from RNA isolated with Trizol (Invitrogen). cDNA levels were measured by quantitative RT-PCR (QPCR) using SYBRgreen (Biorad), and normalized to levels of beta-actin according to the following formula: $100 / 2^{[Ct(\text{gene of interest}) - Ct(\text{beta-actin})]}$. Primer sequences are available upon request.

Chromatin immunoprecipitation and ChIP-Seq

Chromatin immunoprecipitation (ChIP) was carried out as previously described (Dahle et al. 2010). Primer sequences are available upon request. Enrichment was calculated using the following formula: $\text{enrichment relative to input} = 100 / 2^{(Ct(\text{IP}) - Ct(\text{input}))}$. Ct(IP) is the threshold value from QPCR of ChIP'ed DNA and Ct(input) is the threshold value of input

DNA. ChIP values from isotype matched unspecific IgG were subtracted from the ChIP values obtained for each specific indicated antibody. Significance ($p < 0.05$) was determined using two-tailed t-test. For ChIP-seq, chromatin immunoprecipitates were sent to Active Motif for commercial deep sequencing and analysis. Average 35 nucleotide sequence reads were mapped to the genome using the ELAND algorithm. Fold H3K27me3 enrichment was determined by measuring and comparing areas under the peaks. The Venn diagram was generated using R (programming language) for statistical analysis, comparing H3K27me3 methylation enrichment genome-wide between the different samples.

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Key Findings

- Smad2/3 antagonizes pluripotency through negative regulation of *Prdm14*
- Smad2/3 counters transcriptional priming of endoderm-specifying genes
- Blocking signaling in pluripotent stem cells enhances endoderm formation

Figure 1. Genome-wide analysis identifies *Prdm14* as a Smad2/3 target negatively regulated by limiting Jarid2-PRC2 and RNAPII accumulation

A: Venn diagram representation of ChIP-seq data. Left panel, genomic sites showing increased H3K27me3 upon inhibition of Smad2/3 signaling. Blue ellipse represents sites identified in pluripotent cells (grown in LIF). Pink ellipse represents those sites identified in differentiated cells (grown without LIF). 222 sites are unique to pluripotent cells, 1332 unique to differentiated cells and 193 found under either condition. Right panel similarly shows genomic sites with decreased H3K27me3 after inhibiting signaling. **B:** ChIP analysis

of H3K27me3 at the *Prdm14* locus. **C:** *Prdm14* mRNA expression. Fold mRNA levels determined by quantitative RT-PCR normalized to beta-actin levels are displayed on the y-axis. **D:** ChIP analysis of Jarid2 binding at *Prdm14*. **E:** ChIP analysis of RNAPII binding at the *Prdm14* locus. For **B** - **E**, error bars display SEM, with n = 3, with p-values shown above. Data from untreated wild type (wt) ESCs shown using light blue bars; for ALKi treated wt cells, dark blue bars. For **C** - **E**, data from untreated *Suz12*^{-/-} ESCs shown using light green bars; for ALKi treated *Suz12*^{-/-} ESCs, dark green bars. For all ChIP experiments, fold change relative to unspecific IgG is displayed on the y-axis.

Figure 2. Enhanced endoderm formation after blocking Smad2/3 in pluripotent cells

A-D: Representative fields of ESCs immunostained for Oct4 and Eomes, and nuclei stained with Hoechst dye. **A** and **B** show undifferentiated cells, with the cells in **B** having been treated with ALKi for 96 hours. **C** and **D** show cells 96 hours after LIF was removed to induce differentiation, with **D** showing cells that were pretreated with ALKi prior to LIF withdrawal. **E:** Quantitation of the extent of endoderm differentiation as the percent of cell types, out of the total cell count determined by counting Hoechst stained nuclei. Cells stained for both Eomes and Oct4 (endoderm-committed cells) shown in yellow; cells

staining only for Eomes (endoderm cells) shown in red. A minimum of 3 fields was counted for each treatment in three independent experiments. Error bars display SEM, with p-values shown to the right.

Figure 3. Enhanced priming of endoderm-specifying genes after blocking Smad2/3 in pluripotent cells

Quantitative RT-PCR determination of mRNA expression, normalized to beta-actin levels, for **A:** *Oct4*; **B:** *Nodal*; **C:** *Eomes*; **D:** *Foxa2*; **E:** *Sox17*; and **F:** *Nestin*. Time points were 24, 48 and 96 hours after LIF withdrawal to induce differentiation. For all experiments, n = 3. Data from untreated wild type (wt) ESCs shown using light blue lines; for ALKi pre-treated wt ESCs, dark blue lines. Data from untreated *Suz12*^{-/-} ESCs shown using light green lines; for ALKi treated *Suz12*^{-/-} ESCs, dark green lines. R (programming language) was used for

statistical analysis. Changes in mRNA expression were analyzed using linear regression with the estimated variance shown in light gray above and below lines. P-values for *Nestin* expression at 96 hours, comparing no treatment vs. ALKi pretreatment, are shown in **F**.

Figure 4. Enhanced priming of endoderm-specifying genes in pluripotent cells after blocking Smad2/3 signaling

ChIP analysis, with fold change relative to unspecific IgG displayed on the y-axis, of H3K27me3 levels (left panels), Jarid2 binding (middle panels) and RNAPII binding (right panels) for **A: Oct4**; **B: Nodal**; **C: Eomes**; **D: Foxa2**; and **E: Nestin**. Data from untreated wild type (wt) ESCs shown using light blue bars; for ALKi treated wt cells, dark blue bars. Data from untreated *Suz12*^{-/-} ESCs shown using light green bars; for ALKi treated *Suz12*^{-/-} ESCs, dark green bars. Error bars display SEM, n = 3, with p-values shown above.

Figure 5. Model for transcriptional priming or enhancement

A: Schematic representation of the *Prdm14* locus in pluripotent ESCs under normal conditions (left), or in the presence of ALKi to block Smad2/3 signaling (right). Normal *Prdm14* expression levels result from Smad2/3 signaling counteracting PRC2 function (left). ALKi treatment leads to enrichment of Jarid2 containing PRC2 complexes, which recruit RNAPII to the locus to augment ongoing transcriptional levels (right). **B:** Schematic representation of an unexpressed endoderm-specifying locus in pluripotent ESCs under normal conditions (top left), with Smad2/3 signaling counteracting PRC2 function.

Following LIF removal to induce differentiation (top right), signaling maintains the locus in a transcriptionally permissive state, with intrinsic lineage specific transcription factors (LS-TF) and the elongating form of RNAPII regulating transcriptional rates. In pluripotent ESCs treated with ALKi (bottom left), enrichment of Jarid2 containing PRC2 complexes leads to recruitment of the paused form of RNAPII. Upon differentiation in the absence of further ALKi treatment (bottom right), restored Smad2/3 signaling counteracts PRC2 repression as normal, and the additionally recruited RNAPII augments expression levels.