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Nodal Signaling Recruits the Histone Demethylase Jmjd3 to Counteract Polycomb-Mediated Repression at Target Genes

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Abstract

Both intercellular signaling and epigenetic mechanisms regulate embryonic development, but it is unclear how they are integrated to establish and maintain lineage-specific gene expression programs. Here, we show that a key function of the developmentally essential Nodal-Smads2/3 (Smad2 and Smad3) signaling pathway is to recruit the histone demethylase Jmjd3 to target genes, thereby counteracting repression by Polycomb. Smads2/3 bound to Jmjd3 and recruited it to chromatin in a manner that was dependent on active Nodal signaling. Knockdown of Jmjd3 alone substantially reduced Nodal target gene expression, whereas in the absence of Polycomb, target loci were expressed independently of Nodal signaling. These data establish a role for Polycomb in imposing a dependency on Nodal signaling for the expression of target genes and reveal how developmental signaling integrates with epigenetic processes to control gene expression.

INTRODUCTION

The development of metazoans is driven by extracellular signaling pathways that establish lineage-specific patterns of gene expression. Although these signals are transient, the genomic programs that they initiate can be stably maintained across multiple cell generations, *yet also* remain responsive to developmental cues (1–3). This transcriptional plasticity is an emergent property that is derived from complex epigenetic regulation largely at the level of chromatin (4). Chromatin is composed of DNA wrapped around histones, which are subject to numerous posttranslational modifications, including acetylation and methylation. These modifications can directly change chromatin structure, and thus the accessibility of DNA, or serve as binding platforms for various transcriptional regulators. As an example of the latter, trimethylation of Lys²⁷ (K27) of histone H3 (H3K27me3), which is carried out by Polycomb repressive complex 2 (PRC2), provides a site for the binding of Polycomb repressive complex 1, which mediates repression of gene expression (1, 5–7). Global analysis of Polycomb-binding profiles has revealed a large number of target genes, which, though predominantly involved in various developmental fate decisions, are also

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SUPPLEMENTARY MATERIALS

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involved in the maintenance of stem cell pluripotency and in cellular homeostasis (1, 5–9). With the recent identification of histone demethylases that target H3K27me3 (10, 11), it is now clear that Polycomb-mediated repression is highly dynamic and thus well suited to providing the transcriptional plasticity necessary for continued responsiveness to developmental signals. However, at present, little is known about how dynamic Polycomb action is linked to that of the major cell-to-cell signaling pathways that control early development.

One of the earliest-acting developmental signaling pathways is initiated by Nodal, a transforming growth factor- β (TGF- β)-related extracellular ligand. The Nodal signaling pathway acts at several points during vertebrate embryogenesis and is essential for a number of developmental processes, including formation of the mesoderm and endoderm and patterning along the anterior-posterior and left-right body axes (12, 13). Nodal is also required for maintaining the pluripotency of human embryonic stem (ES) cells and mouse epiblast stem (EpiS) cells (14–16), but apparently not for mouse ES cells, which instead depend on leukemia inhibitory factor (LIF) (17). Nodal signaling activates the downstream signal transducers Smad2, Smad3, or both (here abbreviated as Smads2/3) (18), which, in complex with co-Smad4, function in the nucleus to regulate gene transcription. *Nodal* is itself a target of this signaling pathway, as are the genes that encode extracellular antagonists of the pathway, which enables the expression of *Nodal* to be strictly controlled by a self-enhancing and self-limiting autoregulatory loop (19).

Here, we demonstrate in mouse ES cells that *Nodal* is targeted for repression by Polycomb and that Nodal signaling regulates the expression of *Nodal* primarily by counteracting the effects of Polycomb. We found that in the absence of Nodal signaling, *Nodal* was bound by Polycomb proteins and accumulated H3K27me3-repressive histone marks. Furthermore, Nodal-activated Smads2/3 interacted with and recruited the H3K27me3 demethylase Jmjd3 to the *Nodal* locus, thereby reversing Polycomb-mediated histone trimethylation. We also established *Brachyury* as a target of Nodal signaling and found that this gene also was regulated by the recruitment of Jmjd3 by Smads2/3. In the absence of Polycomb function, both *Nodal* and *Brachyury* were expressed independently of Nodal signaling. Thus, the transcriptional requirement of Nodal-Smads2/3 signaling was primarily to counteract Polycomb at these loci. In turn, the epigenetic function of Polycomb was necessary for the responsiveness of *Nodal* and *Brachyury* to Nodal-Smads2/3 signaling and for the stable repression of these genes in the absence of signaling. These findings provide new insight into how developmental transcriptional plasticity can derive from the cooperative action of the epigenetic machinery and developmental signaling.

RESULTS

Nodal is a Polycomb target

To explore potential links between the Nodal signaling pathway and epigenetic regulation by Polycomb, we first investigated *Nodal*, which is a well-established target of Nodal-Smads2/3 signaling (20–24). Through chromatin immunoprecipitation (ChIP) assays, we looked for H3K27me3, the modification deposited by PRC2. We analyzed two regulatory regions within the *Nodal* locus (Fig. 1A). The intronic asymmetric enhancer (ASE) contains binding

sites for Smads2/3 and is responsive to Nodal signals (21, 22, 25, 26). The upstream proximal epiblast enhancer (PEE) lacks Smads2/3-binding sites but is responsive to Wnt signals (23, 27). We compared mouse ES cells, which actively express *Nodal*, with B16 melanoma cells, which do not express *Nodal* (fig. S1). In the latter cell type, both the ASE and the PEE contained H3K27me3 at an abundance that was considerably higher than that in ES cells (Fig. 1B, left panel). This suggested that *Nodal* might indeed be targeted by Polycomb and that H3K27me3 might be an epigenetic mark to maintain repression.

To confirm that *Nodal* is a target of Polycomb, we examined chromatin modifications that occurred upon inhibition of the Nodal signaling pathway by treating ES cells with SB431542, a small-molecule inhibitor of the type I Nodal receptors that blocks activation of Smads2/3 (28). The binding of Smads2/3 to the ASE domain, as detected by ChIP assay, was completely lost after treatment with SB431542 for 96 hours (fig. S2). SB431542 also led to a considerable enrichment in H3K27me3 (Fig. 1B) and of the binding of Suz12, an essential component protein of PRC2 (Fig. 1C), primarily at the ASE domain. Control ChIP analysis of total histone H3 revealed a less than twofold difference in overall histone H3 abundance within the PEE and ASE upon treatment with SB431542 (fig. S3A). Thus, the observed increase in the abundance of H3K27me3 was not due to changes in nucleosome density. Although we found substantial reductions in the abundance of *Nodal* messenger RNA (mRNA) in the presence or absence of LIF, SB431542 in the presence of LIF for 96 hours did not lead to any changes in the expression of the genes encoding the pluripotency factors Oct4 and Nanog (Fig. 1D). Thus, recruitment of Polycomb to the *Nodal* locus was due specifically to inhibition of Smads2/3 signaling and concomitant loss of *Nodal* expression and was not an indirect consequence of the differentiation of ES cells. As a negative control, we also examined *Suz12* homozygous mutant (*Suz12*^{-/-}) ES cells, which completely lack PRC2 function (29). As expected, ChIP analyses showed that H3K27me3 was absent and that there was no Suz12 bound at the *Nodal* locus in *Suz12*^{-/-} cells (Fig. 1, B and C, right panels), which confirmed the specificities of the reagents used.

Together, these findings indicated that inhibiting Nodal signaling led to the targeting of PRC2 complexes to the *Nodal* locus and to the deposition of H3K27me3 epigenetic marks that initiate stable gene-silencing events. Thus, we concluded that *Nodal* is a bona fide Polycomb target gene. Polycomb is probably initially recruited to the area surrounding the transcriptional start site, because the extent of binding of Suz12 at the nearby ASE was greater than that at the far upstream PEE. It is likely that, subsequently, the repressive epigenetic H3K27me3 mark spreads throughout the locus, as was seen in B16 cells, facilitating silencing of the locus. The lack of binding of Suz12 at the inactive *Nodal* locus in B16 melanoma cells (Fig. 1C, left panel) was likely due to the locus being constitutively silenced in these cells. Under such conditions, the locus would not be expected to retain PRC2 complexes.

Regulation of *Nodal* expression by Nodal is Polycomb-dependent and Trithorax-independent

Having established that the *Nodal* locus is a target for repression by Polycomb, we predicted that in the absence of Polycomb, *Nodal* expression should not be inhibited upon loss of

Nodal signaling. To test this, we treated *Suz12*^{-/-} ES cells with SB431542 and examined the expression of *Nodal*. The basal abundance of *Nodal* mRNA in *Suz12*^{-/-} cells was similar to that in wild-type cells cultured with or without LIF. However, unlike wild-type cells, treatment with SB431542 for 96 hours did not lead to loss of *Nodal* expression (Fig. 1D). Thus, *Nodal* could not be repressed in the absence of Polycomb, as predicted. Furthermore, this result revealed that Smads2/3 were unnecessary for ongoing transcription of *Nodal* in *Suz12*^{-/-} cells. This suggests that the primary transcriptional role of Smads2/3 is to counteract repression mediated by Polycomb. That Smads2/3 may be otherwise dispensable is surprising given their known interactions with various transcriptional regulatory proteins (30). However, it is possible that in the context of ongoing transcription at the active *Nodal* locus, they may have a narrower role.

We postulated that one such role might be to recruit Trithorax complexes, which are thought to antagonize the repressive function of Polycomb. ChIP analysis of the Trithorax-mediated histone modification H3K4me3 showed enrichment at the active *Nodal* locus, specifically at the ASE region near the transcriptional start site (Fig. 2A). Consistent with a role in active gene expression, H3K4me3 was not detected at the inactive *Nodal* locus in B16 cells or in ES cells treated with SB431542 for 96 hours in the absence of LIF (Fig. 2A). However, inhibition of Smads2/3 signaling in the presence of LIF only led to a less than twofold reduction in the abundance of H3K4me3 at the ASE (Fig. 2A). Consistent with this, inhibition of Nodal-Smads2/3 signaling in the presence of LIF also did not result in any reduction in the binding within the *Nodal* ASE of Rbbp5 (Fig. 2B) and Ash1 (Fig. 2C), two common component proteins of Trithorax complexes. Together, these data suggest that Nodal-Smads2/3 signaling has only a limited or minimal role in recruiting Trithorax to the *Nodal* locus and in promoting H3K4me3 methylation.

Activated Smads2/3 recruit Jmjd3 to the *Nodal* locus

It has become evident that Polycomb-mediated methylation of histones can be reversed by H3K27me3-specific demethylases (10, 11), including Jmjd3, which is found in ES cells and is essential for differentiation (31). Therefore, we investigated whether Nodal-Smads2/3 signaling might counteract Polycomb by recruiting Jmjd3 to the *Nodal* locus. Through ChIP analyses, we detected endogenous Jmjd3 at the active *Nodal* locus in ES cells (Fig. 3A). The abundance of Jmjd3 was ~30-fold higher at the active *Nodal* locus than at the silenced locus in B16 cells, consistent with Jmjd3 playing a role in maintaining transcriptional activity. Moreover, inhibition of Nodal-Smads2/3 signaling for 96 hours in the presence or absence of LIF eliminated the binding of Jmjd3 at the ASE and significantly lowered it at the PEE ($P < 0.05$), indicating a strict dependence of Jmjd3 presence at the locus on the activation of Smads2/3. Even after only 24 hours of treatment with SB431542 in the absence of LIF, before any effect on the expression of *Nodal* (fig. S4A), the abundance of Jmjd3 at the ASE was already significantly reduced ($P < 0.05$, fig. S4B), and that of H3K27me3 was markedly increased (fig. S4C). Consistent with the recruitment of Jmjd3 by activated Smads2/3, endogenous Smads2/3 coimmunoprecipitated with Jmjd3 (Fig. 3B), but only in the context of active Nodal-Smads2/3 signaling. Treating ES cells with SB431542 eliminated this interaction but did not change the overall abundances of either Jmjd3 or Smads2/3. Thus,

only activated Smads2/3 were capable of interacting with Jmjd3, perhaps because of localization, phosphorylation status, or both.

If Nodal signaling activates gene expression by recruiting Jmjd3, we would expect that in the absence of Jmjd3, target genes would no longer be expressed. To test this prediction, we carried out short hairpin RNA (shRNA)–mediated knockdown of Jmjd3 in wild-type ES cells and examined the effect on the expression of *Nodal* (Fig. 3C). In cells in which Jmjd3 was knocked down by 90%, we found a reduction in the abundance of *Nodal* mRNA by a factor of ~4. This result provides firm support for the idea that Nodal-Smads2/3 signaling regulates the expression of *Nodal* primarily through the recruitment of Jmjd3 to demethylate Polycomb-deposited H3K27me3. Knockdown of Jmjd3 also led to reductions in the expression of both *Nanog* and *Oct4* (fig. S5). Given the absence of any substantial effect on the expression of *Nanog* and *Oct4* in SB431542-treated cells grown in LIF (Fig. 1D), these genes may not be direct targets of Nodal. Rather, their reduced expression in Jmjd3 knockdown cells may instead reflect the importance of Jmjd3 for the pluripotency of ES cells. Consistent with this, we could not increase the numbers of Jmjd3 knockdown cells to the amount necessary for ChIP analyses without the abundance of Jmjd3 returning to that of control cells, presumably as a result of selection for and overgrowth of revertant cells.

Smads2/3 signaling is required to activate the silent *Brachyury* locus in ES cells

To determine whether the recruitment of Jmjd3 by Smads2/3 is a common mechanism for regulating gene activity, we analyzed additional Nodal target genes. For the well-established Nodal targets *Lefty1* and *Lefty2*, their basal mRNA abundances in ES cells were too low to enable us to make any consistent conclusions. We then turned to the *Brachyury* locus, a marker of mesodermal differentiation and a known target of the canonical Wnt– β -catenin signaling pathway (Fig. 4A). Although there was no evidence for direct regulation of the expression of *Brachyury* by Smads2/3 signaling, it seemed likely that this might be the case, given a number of in vivo and in vitro studies that place *Brachyury* downstream of *Nodal* (20, 32, 33). *Brachyury* is transcriptionally inactive in ES cells (Fig. 4B) but can be induced by activating canonical Wnt– β -catenin signaling with BIO, a small-molecule inhibitor of glycogen synthase kinase 3 β (34). Treatment with BIO for 48 hours efficiently induced the expression of *Brachyury* (Fig. 4B) and resulted in significant ($P < 0.05$) β -catenin binding, as detected by ChIP assay. This was seen specifically in the upstream regulatory region (URR) of *Brachyury*, but not 2 kb downstream in the first intron (Fig. 4C). If cells treated with BIO were also treated with SB431542, the induction of *Brachyury* expression was completely blocked (Fig. 4B), showing that transcription requires the Smads2/3 pathway, which is constitutively active in ES cells. Cells treated with BIO and SB431542 still showed β -catenin binding at the *Brachyury* URR (Fig. 4C), indicating that the inhibitory effect of SB431542 on transcription was not through indirect inhibition of the Wnt pathway. Binding of Smads2/3 was detected specifically within the URR and not the intron, by ChIP analysis, but only upon activation of the Wnt pathway with BIO (Fig. 4D). Given that Smads have limited DNA sequence specificity and affinity, it is likely that the recruitment of Smads2/3 to the *Brachyury* locus was mediated by interaction with β -catenin. Consistent with this, we coimmunoprecipitated endogenous β -catenin and Smads2/3 (Fig. 4E). This interaction was strongest in the context of active Wnt and Smads2/3 signaling and was effectively eliminated

in the absence of activated Smads2/3. Together, our data show that *Brachyury* is a target of integrated Nodal-Smads2/3 and Wnt- β -catenin signaling.

Nodal-Smads2/3 signaling activates *Brachyury* by recruiting Jmjd3 to counteract Polycomb

Suz12 binds to *Brachyury* (29) and thus can potentially target the locus for Polycomb-mediated repression. Through ChIP analyses, we found considerable binding of Suz12 in the URR of the inactive locus in untreated ES cells (Fig. 5A). H3K27me3 marks were abundant in the URR and in the downstream intron region (Fig. 5B). Conversely, Jmjd3 was low at both domains (Fig. 5C). In BIO-treated cells that expressed *Brachyury*, both H3K27me3 and Suz12 were reduced in abundance relative to that in untreated cells, whereas the binding of Jmjd3 was significantly increased ($P < 0.05$), with the highest amounts in the URR, the region to which Smads2/3 was bound. Cells treated with both BIO and SB431542, which failed to induce expression of *Brachyury*, had a high abundance of H3K27me3, similar to that found in untreated cells (Fig. 5B), and also showed a significant increase ($P < 0.05$) in the amount of Suz12 bound at the URR (Fig. 5A). The binding of Jmjd3 in cells treated with both BIO and SB431542 was low, similar to that found in untreated cells (Fig. 5C). These treatments had no substantial effect on total H3 ChIP signals at the URR (fig. S3B). Together, these results are consistent with the inhibition of *Brachyury* expression by Polycomb, with β -catenin required to recruit or stabilize the binding of Smads2/3, or both, which in turn engage Jmjd3 to demethylate PRC2-deposited histone marks.

To address whether Nodal-Smads2/3 signaling was required for functions other than the recruitment of Jmjd3 to counteract Polycomb at the *Brachyury* locus, we examined the expression of *Brachyury* in *Suz12*^{-/-} ES cells. We first established that *Brachyury* was not expressed in low-passage *Suz12*^{-/-} ES cells grown under normal conditions and that its expression could be induced with BIO (Fig. 4B, right panel). This indicated that loss of Polycomb-mediated repression in itself was not sufficient to activate the expression of *Brachyury*. We then treated *Suz12*^{-/-} ES cells with BIO and found that induction of the expression of *Brachyury* was as efficient as that in wild-type cells. Unlike in wild-type cells, however, the abundance of *Brachyury* mRNA induced by BIO was not significantly affected by SB431542 treatment (Fig. 4B, right panel). Thus, induction of *Brachyury* expression is not dependent on the Smads2/3 pathway in the absence of Polycomb function. This again points to a primary role for Smads2/3 in counteracting Polycomb and extends this paradigm to include derepression of an inactive gene.

DISCUSSION

The Nodal signaling pathway is used and reused at several critical stages of vertebrate development, differentially regulating the expression of distinct target gene sets in cellular contexts as different as pluripotent stem cells and differentiating mesoderm. Here, we showed that this pathway, in concert with intrinsic and other signal-induced factors, is integrated with Polycomb-mediated epigenetic regulation to impose signaling dependency on target gene activation (Fig. 6). We identified two Nodal target genes as capable of being repressed by Polycomb. In both cases, Nodal signaling regulates their expression by

activating Smads2/3, which in turn recruit the H3K27me3 demethylase Jmjd3 to chromatin to counter repression by Polycomb. Smads2/3 depend on the intrinsic transcription factor Foxh1 for recruitment to the *Nodal* locus to maintain ongoing expression in pluripotent stem cells (35–37). At the *Brachyury* locus, canonical Wnt signaling results in the recruitment of β -catenin, which then recruits Smads2/3 to activate expression. However, in the absence of Polycomb, both genes are freed from their dependency on Nodal-Smads2/3 signaling for their expression. Thus, selective regulation of these two Nodal targets depends on the overall epigenetic state, which includes both the chromatin status of the target gene and the cell type-specific pool of transcriptional cofactors that are available. As demonstrated here, Polycomb-mediated epigenetic repression of the expression of *Nodal* and *Brachyury* imposes a first level of selectivity, enabling activation only in the context of Smads2/3-mediated recruitment of the H3K27me3 demethylase Jmjd3 to alter the chromatin state. The limited DNA binding of Smads2/3 imposes a second level of selectivity, restricting gene activation to developmental contexts in which transcriptional cofactors such as β -catenin or Foxh1 are present.

It is now clear that Polycomb proteins target a large number of genes that control cell fate decisions, with distinct sets of genes targeted in pluripotent cells, differentiated progenitors, and terminally differentiated cells (1, 6, 38). Our findings demonstrate that Nodal-Smads2/3 signaling antagonizes the repression by Polycomb of two target genes with different patterns of expression, one expressed both in pluripotent cells and in differentiated cells and the other restricted to mesodermal progenitors. This raises the possibility that Polycomb-mediated repression may impose signaling dependency on a range of Nodal-Smads2/3 targets expressed across a spectrum of developmental lineages and stages. It will be an important avenue of future research to investigate how widespread this mechanism of gene regulation is.

Given the role of Polycomb-mediated histone methylation in providing cellular memory of transcription states across cell generations, there must exist mechanisms that regulate the activities of histone demethylases (11, 39). The activity of Jmjd3 is regulated by stress-induced transcriptional activation (40–43); however, many cell types, including ES cells, exhibit constitutive expression of the genes that encode these demethylases. Thus, regulation of activity at a different level is required. T-box transcription factors recruit H3K27me3 demethylases to chromatin (44, 45), establishing that activity can be influenced by intrinsic DNA binding factors. Our data extend this finding and show that the activity of Jmjd3 can also be regulated by its controlled recruitment to chromatin by extracellular signals. However, controlling the recruitment of Jmjd3 is not a general feature of all developmental signaling pathways, because Wnt- β -catenin signaling cannot counteract Polycomb-mediated repression of the *Brachyury* locus in the absence of Nodal-Smads2/3 signaling. It remains to be seen whether other developmental signaling pathways can function to recruit histone demethylases to counteract Polycomb at their target genes or whether this is a unique feature of Nodal-Smads2/3 signaling.

We presume that Smads2/3 recruit Jmjd3 to chromatin at the site of Smads2/3 binding. However, for both *Nodal* and *Brachyury*, Jmjd3 is abundant outside of the defined Smads2/3-binding sites in the ASE and URR, respectively, including the *Nodal* PEE region,

which is 13 kb away from the ASE. Given the lack of any binding of Smads2/3 at the PEE (fig. S1B), there must be other, Smads2/3-independent, mechanisms involved in the spread of Jmjd3 from the site of initial recruitment. Similarly, Smads2/3-independent mechanisms likely are involved in the loss of Jmjd3 from chromatin upon cessation of Nodal signaling. After 24 hours of treatment with SB431542 in the presence of LIF, there was almost no change in the abundance of Jmjd3 at the PEE (fig. S4B). There was a substantial decrease in the amount of Jmjd3 at the ASE, but the amounts there were apparently still sufficient to prevent accumulation of H3K27me3. However, in the absence of LIF, Jmjd3 was virtually undetectable at the ASE within 24 hours, explaining the accumulation of H3K27me3. These differences are likely due to mechanisms directly or indirectly linked to LIF signaling, which might involve active expulsion or regulated turnover of Jmjd3 protein. It will be important to further explore the mechanisms that influence the spread of Jmjd3 throughout the locus and the kinetics of the removal of Jmjd3 upon loss of the Nodal signaling.

In summary, our findings extend our understanding of how the chromatin status of genes plays an essential role in determining how they respond to signaling and how signaling pathways influence chromatin composition in turn. By establishing a mechanistic link between Polycomb-mediated repression and Nodal signaling, our study provides new insight into how cooperation between the epigenetic machinery and developmental signaling pathways can confer the transcriptional plasticity that underlies developmental gene regulation.

MATERIALS AND METHODS

Cell culture

Wild-type E14tg2a ES cells were acquired from BayGenomics. *Suz12*^{-/-} ES cells were a gift from K. Helin. ES cells were maintained feeder-free and grown in Knockout Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), LIF (Millipore), Glutamax (Invitrogen), and nonessential amino acids (Invitrogen). B16 melanoma cells were maintained in standard DMEM with 10% FBS. SB431542 (Sigma) was used at 5 μ M, and BIO (EMD Biosciences) was used at 2 μ M.

Antibodies

Antibodies against Suz12, H3K27me3, H3K4me3, unmodified H3, and Jmjd3 were from Abcam. Normal rabbit immunoglobulin G (IgG) and normal mouse monoclonal IgG were from Millipore. Antibody against β -catenin (H-102) was from Santa Cruz Biotechnology. Antibodies against Smads2/3 were from BD Biosciences.

Reverse transcription polymerase chain reaction assays

Complementary DNA (cDNA) was generated with SuperScript III (Invitrogen) from RNA isolated with Trizol (Invitrogen). The abundances of cDNAs were measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) with SYBR Green (Bio-Rad) and normalized to the abundance of β -actin according to the following formula:

$$100/2^{[C_t(\text{gene of interest}) - C_t(\beta\text{-actin})]}. \text{ See table S1 for primer sequences.}$$

ChIP assays

The protocol used was adapted from Lee *et al.* (46). Cells were trypsinized and counted, and cell suspension was adjusted to 1×10^6 cells/ml. Cells were fixed in 1.5% formaldehyde (American Chemical Society–grade, Sigma) or 1% formaldehyde for histone-ChIP assays, with continuous rocking for 10 min at room temperature. Fixation was stopped by addition of glycine to a final concentration of 125 mM, followed by washing twice with cold phosphate-buffered saline. Fixed cells were lysed in three consecutive steps to prepare nuclear extracts. First, cells were lysed in buffer containing 0.5% Triton X-100, 0.5% NP-40, 10% glycerol, 50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 0.5 M EDTA, and complete protease inhibitor cocktail (Roche). We used a volume of lysis buffer that would give a cell density of $\sim 1 \times 10^6$ cells/ml. Pelleted nuclei were washed once with buffer containing 10 mM tris-HCl (pH 8), 150 mM NaCl, 0.5 M EDTA, and complete protease inhibitor cocktail. Finally, the nuclear pellet was lysed in 10 mM tris-HCl (pH 8), 150 mM NaCl, 0.1% sodium deoxycholate, 0.5% *N*-lauroylsarcosine, 0.5 M EDTA, and complete protease inhibitor cocktail. Sonication of chromatin was optimized to generate DNA fragments varying in length from 100 to 1000 base pairs (bp). Triton X-100 was added to sheared chromatin to a final concentration of 1% before centrifugation at 13,200 rpm for 10 min. Supernatant containing soluble chromatin was transferred to fresh tubes, and the concentration was estimated by determining the optical density at 260 nm (OD_{260}). All chromatin samples were adjusted to a final concentration of 1 mg/ml, and 400 μ l of this solution was used for each immunoprecipitation. A 10% aliquot was saved for each sample to use as a control for input DNA. After immuno-precipitation, enriched DNA was purified by phenol-chloroform extraction and ethanol precipitation. Enrichment of specific genomic regions was quantified by quantitative RT-PCR (see table S2 for primer sequences). Enrichment was calculated relative to the input control by the following formula: enrichment relative to input = $100/2^{[C_t(\text{IP}) - C_t(\text{input})]}$. $C_t(\text{IP})$ is the threshold value from PCR of chromatin-immunoprecipitated DNA, and $C_t(\text{input})$ is the threshold value from the same PCR analysis of input DNA. The efficiency of the PCR was determined for every primer pair by performing a dilution curve, and only pairs with $\sim 100\%$ efficiency were used. Finally, ChIP values from isotype-matched unspecific IgG were subtracted from the ChIP values from specific antibodies. Statistical analysis was performed with the Student's *t* test on paired data sets to calculate two-tailed *P* values.

Coimmunoprecipitations and Western blotting analysis

Cells were lysed in buffer containing 0.5% Triton X-100, 10% glycerol, 50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 0.5 M EDTA, and complete protease inhibitor cocktail (Roche). Protein lysate (1 mg) was incubated for 2 hours with 1 μ g of primary antibody followed by a 2-hour incubation with protein G–conjugated magnetic beads (Dynabeads, Invitrogen). Immunoprecipitated proteins were detected by Western blotting with the indicated primary antibodies and horseradish peroxidase–conjugated light chain secondary antibodies against rabbit or mouse antibodies (Jackson ImmunoResearch).

shRNA-mediated knockdown of Jmjd3

ES cells were infected with lentivirus expressing control shRNA (no target; SHC002V, Sigma) or Jmjd3 shRNA (TRCN0000095265, Sigma), as previously described (40). Puromycin-resistant colonies were analyzed for knockdown of *Jmjd3* and *Nodal* from 14 to 21 days after infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Polycomb represses *Nodal* upon inhibition of Smads2/3 signaling. **(A)** Diagram of the *Nodal* locus, indicating the positions of PCR primers used for ChIP analysis (red arrows) and RT-PCR (black arrows). **(B and C)** ChIP analysis of H3K27me3 (B) and Suz12 occupancy (C) at the *Nodal* locus in untreated ES cells (black bars), in ES cells 96 hours after withdrawal of LIF (gray bars), in ES cells treated with SB431542 (5 μ M) for 96 hours in the presence or absence of LIF (red filled bars or red striped bars, respectively), and in B16 melanoma cells (blue bars). ChIP assays were performed on both wild-type (WT) ES cells (left panels) and *Suz12*^{-/-} ES cells (right panels). Error bars represent the SEM; *n* = 3 experiments. **(D)** Quantification of the abundances of *Nodal*, *Nanog*, and *Oct4* mRNAs in WT ES cells (left panels) and *Suz12*^{-/-} ES cells (right panels) as percentages of the total amount of actin mRNA. Cells were treated as in (B) and (C). Error bars represent the SEM; *n* = 3 experiments. **P* < 0.01. ns, not significant.

Fig. 2. Smads2/3 do not recruit core Trithorax components. (A to C) ChIP analysis of H3K4me3 (A) and the Trithorax component proteins Rbbp5 (B) and Ash1 (C) at the Nodal locus in untreated WT ES cells (black bars), in ES cells 96 hours after withdrawal of LIF (gray bars), in ES cells after treatment with SB431542 (5 μ M) for 96 hours in the presence or absence of LIF (red filled bars and red striped bars, respectively), and in B16 melanoma cells (blue bars). Error bars represent the SEM; $n = 3$ experiments.

Fig. 3. Nodal activates Smads2/3 to recruit Jmjd3 to the Nodal locus. **(A)** ChIP analysis of Jmjd3 at the *Nodal* locus in WT ES cells treated as described for Fig. 1. Error bars represent the SEM; $n = 3$ experiments. **(B)** Coimmunoprecipitation of activated Smads2/3 and Jmjd3 in lysates from untreated, WT ES cells (left set of panels). No coimmunoprecipitation was found in lysates from SB431542-treated cells (right set of panels). Samples immunoprecipitated (IP) with nonspecific IgG as a control or with antibodies against Jmjd3 were analyzed by Western blotting (WB) with antibodies against Smads2/3 (top panel) or Jmjd3 (lower panel). Data are representative of four independent experiments with similar results. **(C)** shRNA-mediated knockdown of Jmjd3. The abundances of *Jmjd3* and *Nodal* mRNAs after treatment with control shRNA (gray bars) or Jmjd3-specific shRNA (white bars) are shown. The data represent the average from six clonally distinct colonies from three independent infections with lentiviral vectors, demonstrating a ~90% reduction in the abundance of Jmjd3. Error bars represent the SEM; $n = 3$ experiments.

Fig. 4.

Brachyury is a direct target of Smads2/3 signaling. **(A)** The *Brachyury* locus contains elements responsive to Wnt signaling in the 5' URR. Positions of the primers used for ChIP analysis (red arrows) and RT-PCR (black arrows) are indicated. 3'UTR, 3' untranslated region. **(B)** Abundance of *Brachyury* mRNA in WT ES cells (left panel) and *Suz12*^{-/-} ES cells (right panel). Cells were left untreated (black bars), treated with BIO (2 μ M) for 48 hours (green bars), treated with SB431542 (5 μ M) for 48 hours (green stippled bars), or treated with both BIO (2 μ M) and SB431542 (5 μ M) for 48 hours (green striped bars). Active (+) or inactive (-) Nodal and Wnt signaling as a consequence of these treatments is indicated under the graph. Error bars represent the SEM; $n = 3$ experiments. * $P < 0.01$. **(C)** and **(D)** ChIP analysis of the binding of β -catenin (C) and Smads2/3 (D) at the *Brachyury* URR. WT ES cells were left untreated (black bars), treated with BIO alone (green bars), or treated with BIO and SB431542 (green striped bars). Cells were analyzed after 48 hours of treatment. Error bars represent the SEM; $n = 3$ experiments. **(E)** Coimmunoprecipitation of β -catenin and Smads2/3. WT ES cells were left untreated, treated with BIO alone, or treated with BIO and SB431542 for 48 hours. Samples immunoprecipitated with nonspecific IgG as a control or with antibodies against Smads2/3 were analyzed by Western blotting with antibodies against β -catenin (top panel) or Smads2/3 (lower panel). Data are representative of three independent experiments with similar results.

Fig. 5. Nodal-Smads2/3 signaling counteracts Polycomb-mediated repression at *Brachyury* by recruiting Jmjd3. (A to C) ChIP analyses of Suz12 (A), H3K27me3 (B), and Jmjd3 (C) at the *Brachyury* locus. WT ES cells were left untreated (black bars), treated with BIO alone (green bars), or treated with BIO and SB431542 (green striped bars). Cells were analyzed after 48 hours. Error bars represent the SEM; $n = 3$ experiments. * $P < 0.01$.

Fig. 6. Model for the Jmjd3-dependent activation of Polycomb-repressed Smads2/3 target genes. Nodal signaling activates Smads2/3, which are recruited to target loci or are stabilized at target loci, or both, either by intrinsic transcription factors, such as Foxh1 for *Nodal*, or by signal-induced factors, such as Wnt-activated β -catenin for *Brachyury*. Smads2/3 then recruit Jmjd3 to chromatin, resulting in demethylation of H3K27me3. This initiates derepression, which involves the subsequent recruitment of Trithorax group complexes (TrxG) and the general transcriptional machinery (GTM), ultimately resulting in gene activation.