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BMP4 Signaling Acts via Dual-Specificity Phosphatase 9 to Control ERK Activity in Mouse Embryonic Stem Cells

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SUMMARY

Extrinsic BMP and LIF signaling collaboratively maintain mouse embryonic stem cell (ESC) pluripotency, whereas appropriate ERK activity is essential for ESC fate commitment. However, how the extrinsic signals restrain appropriate ERK activity remains elusive. Here, we show that, whereas LIF sustains relatively high ERK activity, BMP4 can steadily attenuate ERK activity by upregulating ERK-specific dual-specificity phosphatase 9 (DUSP9). This upregulation requires Smad1/5 and Smad4 and specifically occurs to DUSP9, but not other DUSPs, and only in ESCs. Through DUSP9-mediated inhibition of ERK activity, BMP signaling reinforces the self-renewal status of mouse ESCs together with LIF. Upon LIF withdrawal, ESCs spontaneously undergo neural differentiation, during which process DUSP9 can partially mediate BMP inhibition on neural commitment. Collectively, our findings identify DUSP9 as a critical mediator of BMP signaling to control appropriate ERK activity critical for ESC fate determination.

INTRODUCTION

Understanding the detailed molecular mechanisms that govern embryonic stem cell (ESC) fate of self-renewal versus differentiation has been an urgent task for both basic research and clinical practices. Mouse ESC fate decision could be controlled both internally and externally. Internally, several core transcription factors, such as NANOG, POU5F1 (OCT4), and SOX2, are regarded to form autoregulatory transcriptional circuits that play pivotal roles in maintaining ESC self-renewal and pluripotency (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008). Recent studies have also highlighted the epigenetic processes (DNA methylation, histone methylation, and so on) as important internal regulation mechanisms of ESC fate determination (reviewed in Bibikova et al., 2008). One of the most impressive internal regulator identified is the extracellular signal-regulated kinase (ERK)—an intracellular signaling mediator of mitogen-activated protein kinase (MAPK) pathways, which is widely involved in growth and differentiation of various types of cells (Kolch, 2005; Silva and Smith, 2008; Ying et al., 2008). Inhibition of ERK activity can promote mouse ESC self-renewal while blocking differentiation by upregulation of core transcription factors like NANOG and TBX3 (Lanner et al., 2010; Niwa et al., 2009). Consistently, overexpression of activated H-Ras, which augments ERK activity, can induce ESC differentiation into primitive endoderm (Yoshida-Koide et al., 2004). Furthermore, ERK activity was recognized as the trigger to induce ESC transition from the self-renewal status into a state more sensitive to differentiation cues (Kunath et al., 2007). The most striking evidence highlighting the importance of ERK in ESC fate determination is that mouse ESC pluripotency can be maintained by specific small molecule inhibitors of ERK and glycogen synthase kinase 3 (GSK3) in the absence of serum or feeder cells (Ying et al., 2008).

Several extracellular cytokine-induced signaling pathways such as leukemia inhibitory factor (LIF), transforming growth factor β (TGF-β), and Wnt signaling have been demonstrated to play critical roles in mouse ESC fate determination (Niwa et al., 1998; Sato et al., 2004; Ying et al., 2003a). Among them, functions of LIF and bone morphogenetic protein (BMP, a member of TGF-β superfamily) as the most classic extrinsic signals have been firmly established. LIF has been routinely added into the culture medium to maintain mouse ESC pluripotency mainly through activation of downstream STAT3 (Niwa et al., 1998). In the absence of serum or feeder cells, mouse ESCs can be maintained by LIF in combination with BMP4 (Ying et al., 2003a).

BMP transduces its signal by binding to its transmembrane type I and type II receptor kinases, which then activate the intracellular receptor-regulated Smad (R-Smad: Smad1, 5, and 8) by phosphorylation. Activated R-Smads are then complexed with co-Smad (Smad4) and together translocated into the nucleus to regulate target gene expression (Datto and Wang, 2000;

Feng and Derynck, 2005; Massagué and Chen, 2000; Massagué et al., 2005; ten Dijke and Hill, 2004). In mouse ESCs, BMP signaling can promote self-renewal by inhibition of differentiation through a cohort of downstream targets like *Id* and other genes (Fei et al., 2010a; Ying et al., 2003a). BMP has also been implicated to function in various lineage commitments (Fei and Chen, 2010; Seuntjens et al., 2009; Watabe and Miyazono, 2009).

Although ESC fate choices can be intricately determined by these internal and external factors, how the two levels of regulation are linked, in particular, how external BMP/LIF and internal ERK is interconnected, in ESC fate decisions remains elusive. In the present study, we report that BMP can steadily inhibit ERK activity through induction of dual-specificity phosphatase 9 (DUSP9, also known as MKP-4). DUSP9, an ERK-specific phosphatase, is a member of the dual-specificity (threonine/tyrosine) phosphatase superfamily, which can dephosphorylate and thus decrease the activities of differential subsets of MAPKs depending on their targeting preference (Jeffrey et al., 2007; Patterson et al., 2009). We further show that DUSP9, as the transcriptional target of BMP signaling, acts as a factor to link extrinsic BMP stimulus to intrinsic ERK activity during ESC fate determination.

RESULTS

BMP Inhibits ERK Activity in Mouse ESCs

In serum- and feeder-free culture, extrinsic signals from BMP and LIF together sustain mouse ESC self-renewal whereas intrinsically ERK activity serves as the primary trigger to promote ESC differentiation (Ying et al., 2003a, 2008). However, whether extrinsic BMP and/or LIF signals coordinate with intrinsic ERK activity is unclear. To address this question, we separately treated R1 mouse ESCs with 10 ng/ml BMP4 or 10 ng/ml LIF and then examined ERK activities by monitoring ERK phosphorylation as the readout. As shown in Figure 1A, we found that there was a slight increase of p-ERK1/2 level after BMP4 was added for 2–4 hr, which was consistent with the previous report by Ying et al. (2003a). However, when BMP4 treatment was extended to 12 hr, ERK activity was significantly decreased. Different from BMP4, LIF induced an immediate strong increase of p-ERK1/2 level at 10 min, and when the treatment time was prolonged, ERK activity was maintained at a steadily higher level although not as strong as that at 10 min (Figure 1B). This is consistent with the previous report that LIF has a stimulating effect on ERK activity (Ying et al., 2003a). We further tested whether BMP inhibition of ERK activity is dependent on LIF signaling. Figure 1C showed that although ERK activity in the presence of LIF was higher than that in the absence of LIF, BMP could inhibit ERK phosphorylation in both contexts, indicating that BMP inhibition of ERK activity is independent of LIF signaling.

It has been shown that autocrine FGF4 is the major source of stimuli to activate ERK in mouse ESCs (Kunath et al., 2007; Stavridis et al., 2007). By use of FGF4 ligand and the FGF receptor inhibitor SU5402, we found that BMP could also inhibit FGF signaling-promoted ERK activity (Figure 1D). These data strongly indicated that inhibition of ERK activity is a direct effect of BMP signaling, but not indirectly through crosstalk with other ERK-interfering signal cascades.

To determine whether BMP inhibition of ERK activity was specific across MAPK family, we examined the phosphorylation levels of other two MAPK members (p38 and JNK) upon BMP treatment and found that neither of them was steadily decreased (Figure S1A available online), indicating that the inhibitory effect of BMP is specific to ERK. Consistent with the decrease of ERK activity, the expression of ERK target genes was decreased significantly upon BMP4 treatment (Figures 1E and S1B). This is in accordance with the inhibitory effect of BMP on the ERK-responsive luciferase reporter pFR-Luc/pFA2-Elk1 (Figure 1F). We then asked whether BMP inhibition of ERK activity is specific for ESCs. Two somatic mouse cell lines (primary mouse embryonic fibroblast [MEF] and NMuMG cells) were treated with 10 ng/ml BMP4, and ERK phosphorylation was examined. In contrast to that in ESCs, ERK activity was not influenced by BMP in these somatic cells (Figure S1C). Thus, our results strongly indicated that BMP signaling inhibits ERK activity specifically in ESCs.

BMP4 Specifically Upregulates DUSP9 in Mouse ESCs

We then tried to address the mechanism underlying the BMP-mediated inhibition of ERK activity. Because ERK is directly activated by its upstream kinase MEK1, we first examined whether BMP influences MEK1 activity. As shown in Figure S1A, the phosphorylation level of MEK1 was unchanged by BMP4, indicating that BMP inhibits ERK activity at the level downstream of MEK1, possibly of ERK itself. As shown in Figure 1A, inhibition of ERK phosphorylation became apparent only after 8 hr of BMP4 treatment. This delayed inhibition led us to hypothesize that BMP may inhibit ERK activity through a transcription-dependent mechanism. To determine whether BMP inhibition of ERK needs de novo protein synthesis, we employed cycloheximide (CHX) to block protein translation for 12 hr in the absence or presence of BMP4 and then assessed ERK phosphorylation. When protein synthesis was blocked by CHX, the ERK protein levels were decreased (Figure 2A). However, ERK activity was no longer reduced by BMP4, indicating that de novo protein synthesis is required for BMP-mediated ERK inhibition.

Because BMP signaling regulates target gene expression through the Smad pathway, we then searched for the possible BMP/Smad target genes, which may account for ERK inhibition. By scrutinizing the candidates identified in our previous work by chromatin immunoprecipitation (ChIP) and ChIP-seq in mouse ESCs (Fei et al., 2010a), we found that both Smad1/5 and Smad4 bound to the promoter region of *Dusp9* gene, which encodes a dual-specificity phosphatase with preference to target ERK1/2 (Figure 2B; Jeffrey et al., 2007; Patterson et al., 2009). *Dusp9* gene also presented in the list of top BMP4-upregulated genes in the previous expression array data (Figure 2C; Fei et al., 2010a). That no other ERK-specific phosphatases were found in the high-throughput data led us to speculate that *Dusp9* is the most promising candidate target to mediate BMP inhibition of ERK.

By using quantitative RT-PCR, we observed a time-dependent induction of *Dusp9* mRNA by BMP4 in R1 ESCs (Figure 2D), and upregulation of DUSP9 protein was also confirmed by immunoblotting (Figure 2E). To exclude the cell strain-specific effect, we confirmed BMP upregulation of *Dusp9* in another mouse ESC line (E14 cells), and similar results were

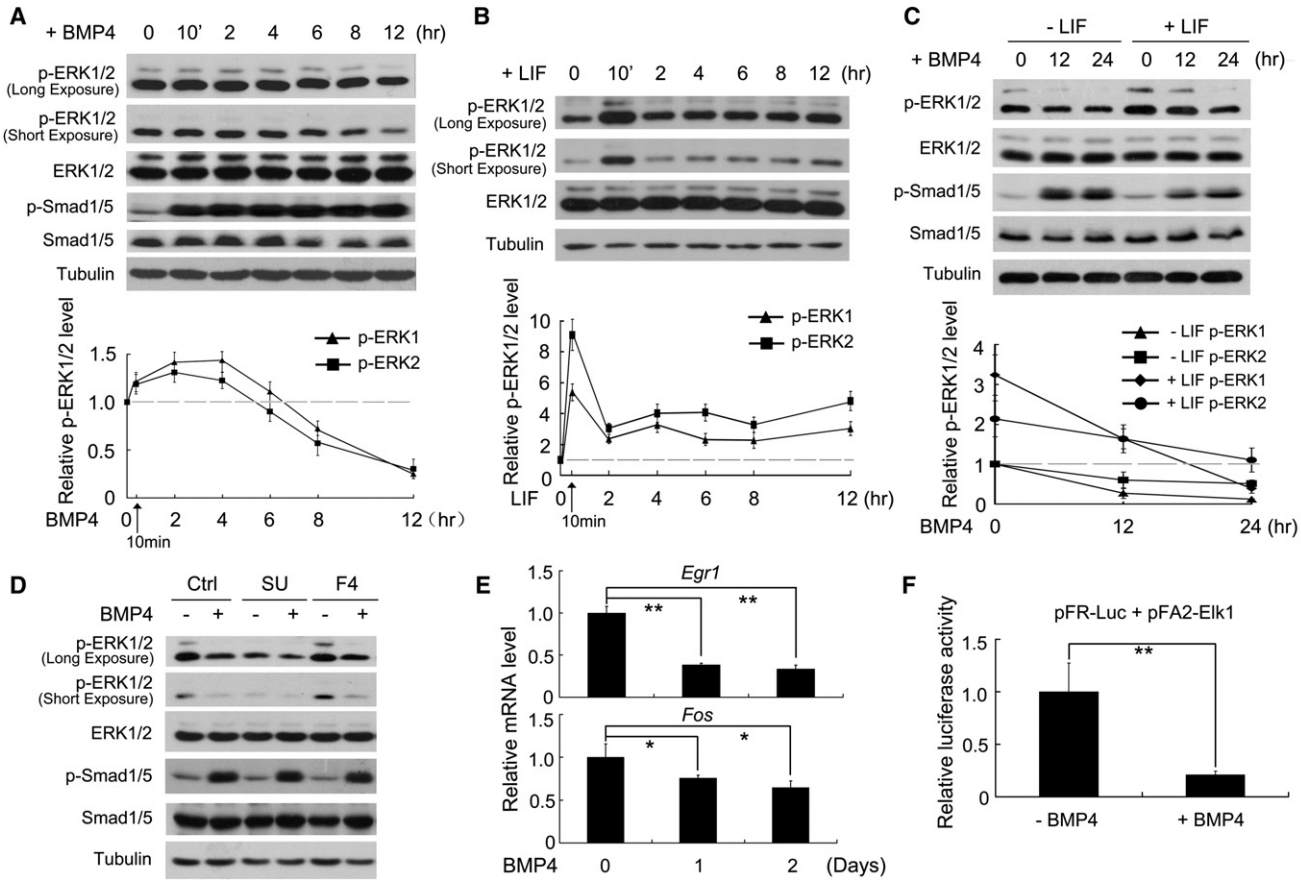

Figure 1. BMP Inhibits ERK Activity in Mouse ESCs

(A) ERK activity is reduced in mouse ESCs upon BMP4 treatment for more than 6 hr. R1 cells cultured in N2B27 medium overnight were treated with BMP4 for the indicated time before being harvested for immunoblotting. Tubulin was used as a loading control.

(B) ERK activity is activated by LIF. R1 cells were treated with LIF for the indicated time before being harvested for immunoblotting.

(C) BMP inhibition of ERK activity is independent of LIF signaling. R1 cells were treated with BMP4 in the presence or absence of LIF before being harvested for immunoblotting.

Levels of p-ERK1/2 were quantified in the lower panel of (A)–(C) and shown as mean ± SEM (n = 3).

(D) BMP inhibits FGF-enhanced ERK activity. R1 cells were treated with 25 ng/ml FGF4, 2 μM SU5402, or 10 ng/ml BMP4, as indicated, for 12 hr before being harvested for immunoblotting. Ctrl, control; SU, SU5402; F4, FGF4.

(E) BMP inhibits ERK target gene expression in ESCs. R1 cells were treated with BMP4 before being harvested for qRT-PCR for *Egr1* and *Fos* expression.

(F) ERK-responsive pFR-luc/pFA2-Elk1 luciferase reporter expression is reduced by BMP4. R1 cells transfected with pFR-luc, pFA2-Elk1, and pRenilla-TK plasmids were treated with or without BMP4 for 24 hr before being harvested for luciferase assay.

Data are shown as mean ± SEM (n = 3) (**$p < 0.01$). See also Figure S1.

obtained (Figures S2A and S2B), supporting the idea that *Dusp9* is a BMP target gene in mouse ESCs. Consistent with our observation that BMP specifically inhibits ERK activity in ESCs but not in somatic cells (Figures 1 and S1C), *Dusp9* was not upregulated by BMP4 in primary MEF and NMuMG cells, although the classic BMP target gene *Id1* could be upregulated in all these tested cells (Figures 2F and S2C–S2E). The specific upregulation of DUSP9 by BMP4 was also evidenced by the finding that activin A, another TGF-β superfamily member, could not induce DUSP9 (Figure 2G). Furthermore, we determined whether other DUSP family members with ERK targeting activities could be upregulated upon BMP4 treatment. As shown in Figure 2H, among the DUSP family members examined, only *Dusp9* mRNA level was significantly enhanced by BMP4. These data together indicated that BMP signaling specifically upregulates the ERK-targeting phosphatase DUSP9 in mouse ESCs.

DUSP9 Mediates BMP Inhibition of ERK Activity

Because DUSP9 is known as an ERK-preferred phosphatase (Jeffrey et al., 2007; Patterson et al., 2009; Theodosiou and Ashworth, 2002) and BMP signaling can upregulate DUSP9 expression, we reasoned that DUSP9 may mediate the inhibitory function of BMP signaling on ERK activity. By overexpressing wild-type DUSP9 in R1 cells, we found that ERK activity was indeed attenuated in a dose-dependent manner (Figure 3A). In accordance, the ERK target genes (*Egr1* and *Fos*) were downregulated by DUSP9 overexpression (Figure 3B).

Then we tried to confirm this with loss-of-function approaches. First, we employed a dominant-negative form of

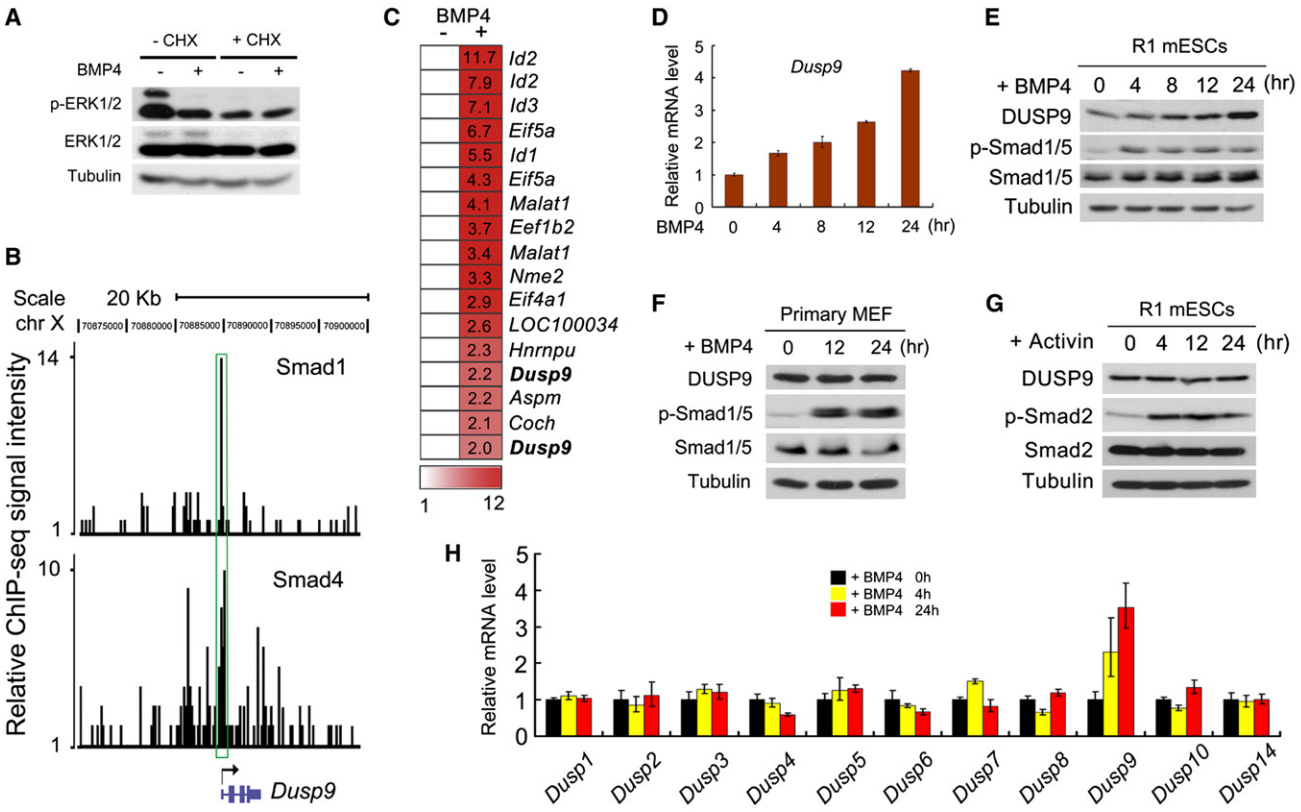

Figure 2. BMP4 Specifically Upregulates DUSP9 in Mouse ESCs

(A) New protein synthesis is required for BMP inhibition of ERK activity. R1 cells were treated with BMP4 for 12 hr in the presence or absence of 2 μg/ml CHX before being harvested for immunoblotting. Tubulin was used as a control.

(B) Smad1/5 and Smad4 bind to the mouse *Dusp9* promoter as identified by ChIP-seq. Green box indicates the binding signal peaks.

(C) Gene expression array heat map of BMP-upregulated genes upon BMP4 treatment for 4 hr in R1 cells. The signal change folds of gene probes are depicted.

(D and E) DUSP9 is upregulated by BMP4. R1 cells were treated with BMP4 and harvested for qRT-PCR for *Dusp9* expression (D) or immunoblotting (E). Relative mRNA levels are shown as mean ± SEM (n = 3).

(F) DUSP9 protein levels are not influenced by BMP4 in MEFs. MEFs were cultured in DMEM medium plus 0.2% FBS overnight and then treated with BMP4 for the indicated time before being harvested for immunoblotting.

(G) DUSP9 protein levels are not affected by activin A. R1 cells were treated with 25 ng/ml activin A before harvested for immunoblotting.

(H) Only DUSP9, but not other DUSPs, is induced by BMP4. R1 cells were treated with BMP4 for the indicated time before harvested for qRT-PCR. Data are shown as mean ± SEM (n = 3).

See also Figure S2.

DUSP9 (dn-DUSP9), in which the critical amino acid cysteine within the catalytic site was mutated to serine (C358S) (Liu et al., 2009; Matsuguchi et al., 2001; Rutter et al., 1995) to block endogenous DUSP9 function. When dn-DUSP9 was expressed, basal ERK activity was increased and the inhibition of ERK activity by BMP4 was completely abolished (Figure 3C). Consistently, at the target gene level, BMP4 could no longer repress *Egr1* and *Fos* expression when dn-DUSP9 was introduced (Figure 3D). Second, we tried to knock down *Dusp9* by RNA interference. Although it has been reported that ERK phosphorylation does not always exhibit expected increase when DUSP9 expression was eliminated by knockdown or knockout because of potential compensation or adaptation effects (Caunt et al., 2008; Christie et al., 2005), we found that two specific small interfering RNA (siRNA) oligos could effectively attenuate DUSP9 expression without significant compensation effects in R1 ESCs (Figure 3E and data not shown). As shown in Figure 3F, knockdown of *Dusp9* enhanced the basal levels of ERK phosphorylation. Importantly, upon depletion of DUSP9, BMP could not inhibit ERK phosphorylation. To further show the functional specificity of DUSP9 in mediating BMP inhibition of ERK activity, we knocked down another DUSP family member, DUSP7, which was previously reported to be highly expressed in mouse ESCs and contributed to ESC self-renewal maintenance (Abujarour et al., 2010). Similar to DUSP9 knockdown, DUSP7 knockdown enhanced the basal ERK phosphorylation, but unlike DUSP9, DUSP7 knockdown did not interfere with BMP inhibition of ERK activity (Figures 3E and 3F). Similar results were also obtained in E14 mESCs (Figure S3). By using immunostaining method, we also observed a reverse correlation between DUSP9 expression and ERK phosphorylation level upon BMP4 treatment (Figure 3G). Taken together, DUSP9 is an essential mediator of BMP inhibition of ERK activity in mouse ESCs.

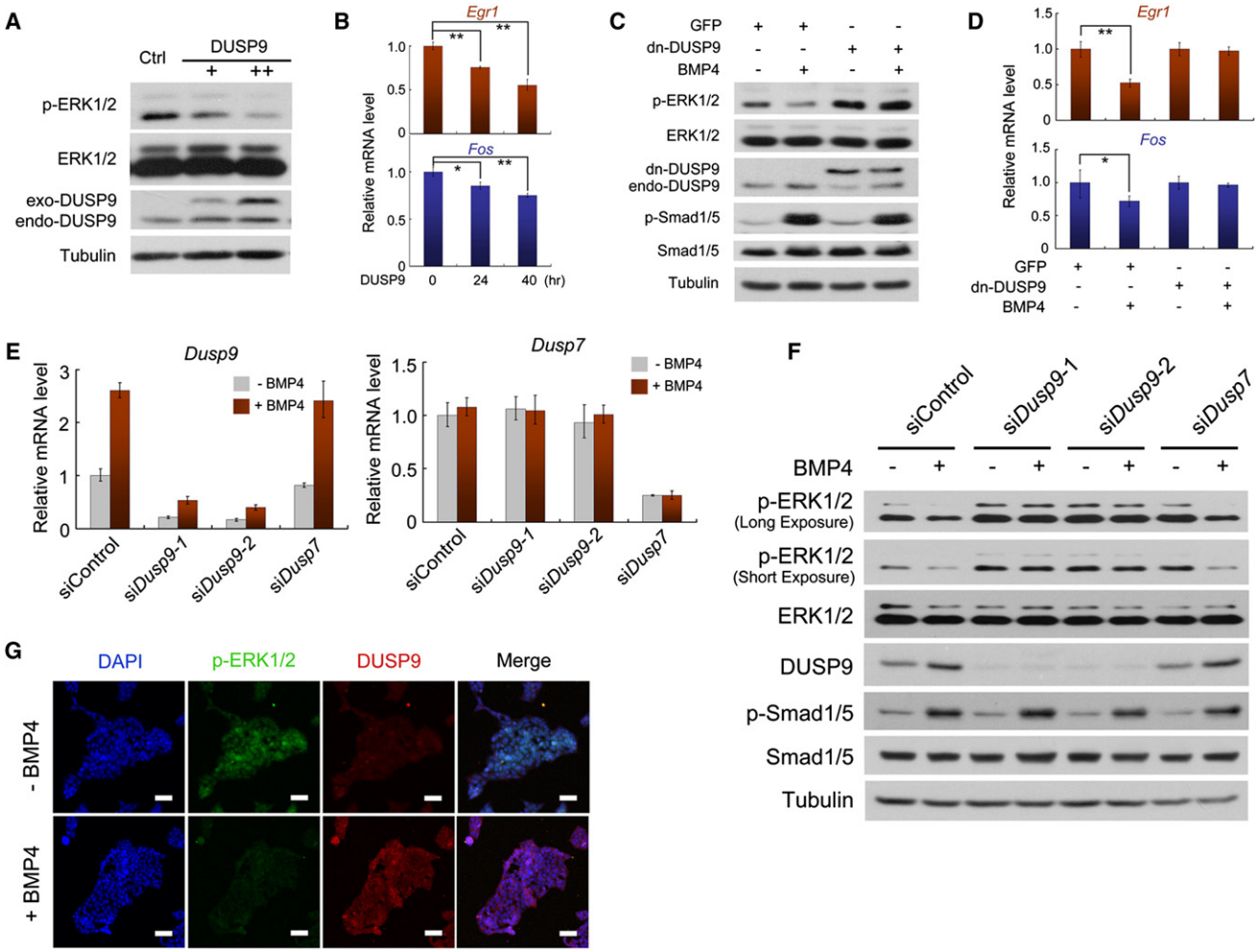

Figure 3. DUSP9 Mediates the Inhibitory Effect of BMP on ERK Activity

(A) Overexpression of DUSP9 inhibits ERK activity in a dose-dependent manner. R1 cells were transfected with different doses of DUSP9 and at 36 hr post-transfection, cells were harvested for immunoblotting.

(B) Overexpression of DUSP9 inhibits ERK target gene expression. R1 cells were transfected with DUSP9 and then harvested at the indicated time for qRT-PCR analysis.

(C) dn-DUSP9 abolishes the inhibitory effect of BMP on ERK activity. R1 cells were transfected with dn-DUSP9 or GFP. At 24 hr posttransfection, the cells were treated with or without BMP4 for 12 hr before being harvested for immunoblotting.

(D) dn-DUSP9 eliminates BMP4 inhibition on expression of the ERK targets *Egr1* and *Fos*. R1 cells were treated the same as in (C). Expression of *Egr1* and *Fos* were analyzed by qRT-PCR after BMP treatment for 24 hr.

(E) *Dusp9* and *Dusp7* are effectively knocked down by siRNA. R1 cells were transfected with siRNA against *Dusp9* or *Dusp7* as indicated. At 36 hr post-transfection, cells were treated with or without BMP4 for 12 hr and then harvested for qRT-PCR.

(F) Knockdown of *Dusp9*, but not *Dusp7*, abolishes BMP4 inhibition of ERK activity. R1 cells were treated the same as in (E) and harvested for immunoblotting.

(G) Reverse correlation of p-ERK1/2 level and DUSP9 expression upon BMP4 treatment in R1 cells. Cells treated with or without BMP4 for 12 hr were immunostained with the antibodies against p-ERK1/2 (green) and DUSP9 (red). The nucleus was counterstained with DAPI (blue). Scale bars represent 50 μm. Data in (B), (D), and (E) are shown as mean ± SEM (n = 3) (*$p < 0.05$; **$p < 0.01$). See also Figure S3.

BMP Upregulation of DUSP9 Is Smad Dependent

After establishing the functional significance of DUSP9 in mediating BMP inhibition of ERK activity, we tried to elucidate the mechanism by which BMP upregulates DUSP9 expression. Because both Smad1/5 and Smad4 interact with the *Dusp9* promoter in our ChIP-seq data (Figure 2B; Fei et al., 2010a), we thus reasoned that BMP upregulation of DUSP9 requires Smad signaling. By employing our previously established stable *Smad4* knockdown R1 ESCs (Fei et al., 2010a), we found that *Dusp9* mRNA was upregulated by BMP4 only in wild-type R1 cells, but not in *Smad4* knockdown cells (Figure 4A). Similar results were obtained at the protein level by immunoblotting (Figure 4B). Furthermore, ERK activity was no longer inhibited by BMP4 in *Smad4* knockdown cells, indicating that BMP inhibition of ERK depends on Smad4 (Figure 4B). Upregulation of *Dusp9* mRNA also requires Smad1/5 as *Smad1* knockdown in *Smad5*$^{-/-}$ TC-1 mouse ESCs completely abolished *Dusp9* induction by BMP4 (Figures 4C and S4A).

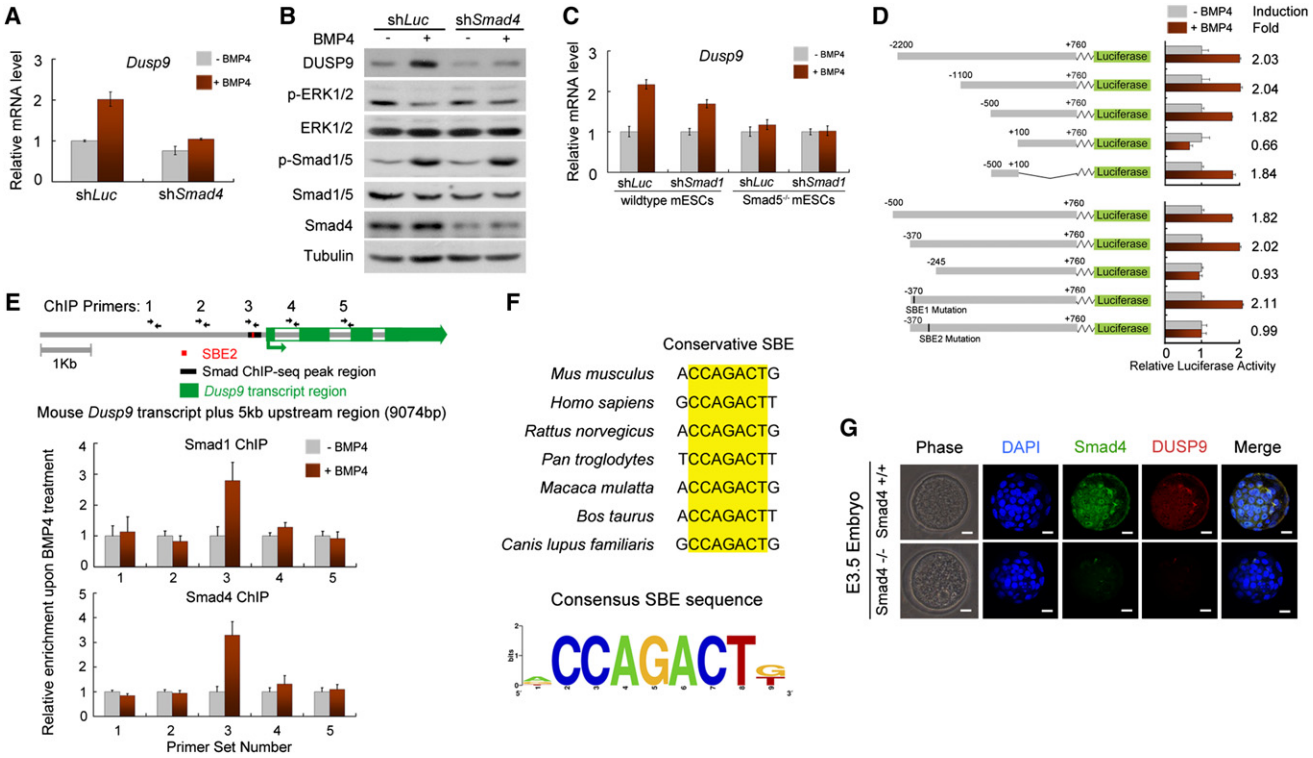

Figure 4. Smad1/5 and Smad4 Are Required for BMP-Induced Expression of DUSP9

(A) *Smad4* knockdown abolishes *Dusp9* induction by BMP4. R1 cells stably expressing shRNA against *luciferase* (sh*Luc*) or *Smad4* (sh*Smad4*) were treated with BMP4 for 12 hr, and *Dusp9* expression was examined by qRT-PCR.

(B) *Smad4* knockdown abolishes DUSP9 protein upregulation and ERK inhibition mediated by BMP4. Control cells or *Smad4* knockdown cells were treated with BMP4 for 12 hr before being harvested for immunoblotting.

(C) Upregulation of *Dusp9* mRNA by BMP4 depends on Smad1/5. Wild-type or *Smad5*$^{-/-}$ TC-1 mouse ESCs transfected with control or *Smad1* shRNA were treated with BMP4 for 12 hr before being harvested for qRT-PCR.

(D) A Smad-binding element in the *Dusp9* promoter is essential for the transcriptional response of BMP4. R1 cells were transfected with the *Dusp9* promoter reporter constructs, treated with or without BMP4 for 24 hr, and harvested for luciferase activity determination. Schematic representation of promoter constructs is shown on the left, and relative luciferase activities shown on the right.

(E) Smad1 and Smad4 specifically bind to the SBE2 region in the *Dusp9* promoter upon BMP4 treatment. ChIP assays were performed with the antibodies against Smad1 or Smad4 in R1 cells treated with or without BMP4 for 6 hr. The immunoprecipitated DNA was amplified by quantitative PCR with the primers detecting specific promoter regions denoted in the upper panel.

(F) The SBE2 found in the *Dusp9* promoter is conserved across mammalian species. Yellow box indicates the conservative SBE motif. The CCAGACT consensus SBE motif was generated by WebLogo.

(G) DUSP9 is undetectable in *Smad4*$^{-/-}$ mouse embryos. Wild-type or *Smad4*$^{-/-}$ E3.5 mouse embryos were immunostained with the antibodies against Smad4 (green) and DUSP9 (red). DAPI counterstained the nucleus (blue). Scale bars represent 20 μm.

Data in (A) and (C)–(E) are shown as mean ± SEM (n = 3). See also Figure S4.

To further investigate the transcriptional regulation of *Dusp9* by the BMP/Smad signaling, the putative *Dusp9* promoter covering the upstream –2200 base pair (bp) to downstream +760 bp region around the transcriptional start site (TSS) was cloned into pGL3 luciferase reporter plasmid. Reporter assay revealed that this region responded well to BMP4 in mouse ESCs (Figure 4D). To map the Smad-responsive element, serial promoter truncations were generated to test for BMP4 responsiveness. We found that the region of −370 bp to −245 bp was the minimal responsive region for BMP4 (Figure 4D). By scrutinizing the minimal responsive 125 bp region, two putative Smad binding elements (SBE) were found, designated as SBE1 and SBE2, respectively (Figure S4B). We then generated single-nucleotide mutations within the two SBEs (Figure S4B) and analyzed their responsiveness to BMP4. Although SBE1 mutation had no effect, SBE2 mutation totally abolished the responsiveness to BMP4 (Figure 4D). We further employed ChIP assay to confirm the binding of Smads to the *Dusp9* promoter. Five pairs of primers around SBE2 region with 1 kb apart from each other were designed to amplify the Smad-interacting DNA, and the results showed that both Smad1 and Smad4 were recruited to the SBE2 region (primer 3) upon BMP4 treatment (Figure 4E). In agreement with this, the SBE2 region was within the Smad1/5- and Smad4-enriched sequence revealed by ChIP-seq analysis (Figure S4C; Fei et al., 2010a). These results indicated that in mouse ESCs, BMP induces the recruitment of Smad1/5 and Smad4 to the *Dusp9* promoter region and induces its expression.

Comparison of the putative *Dusp9* promoter region (5000 bp upstream of TSS) from several mammalian species uncovered

the conserved SBE2 (CCAGACT) sequence in the *Dusp9* promoter of these species (Figure 4F). This absolute conservation further signifies the importance of this transcriptional regulation during evolution.

We also examined whether the BMP/Smad/DUSP9 axis exists during early mouse embryonic development. We stained wild-type and *Smad4*$^{-/-}$ E3.5 embryos with the antibodies specifically against Smad4 and DUSP9, respectively. As shown in Figure 4G, DUSP9 expression was detected only in wild-type but not in *Smad4*$^{-/-}$ embryos, indicating the importance of BMP upregulation of DUSP9 during early mouse embryonic development.

DUSP9 Can Substitute BMP4 to Sustain ESC Self-Renewal in Combination with LIF

As reported previously, mouse ESCs could be maintained by LIF plus BMP4, and withdrawal of BMP4 drove ESC differentiation as shown by cell morphology and alkaline phosphatase (AP) staining (Figure 5A). Because complete inhibition of ERK activity by two or three inhibitors is sufficient to block ESC differentiation (Ying et al., 2008), here we tried to assess whether decrease of ERK activity could substitute BMP4 to maintain ESC self-renewal. Toward this purpose, MEK1 inhibitor PD184352 and FGF receptor inhibitor SU5402 were separately used. Either inhibitor could efficiently inhibit ERK activity in mouse ESCs to an extent similar to BMP4 treatment (Figures S5A and S5B). When PD184352 or SU5402 was added in combination with LIF, ESCs could be effectively maintained in undifferentiated state (Figure 5A), phenocopying those under the LIF plus BMP4 condition.

We further characterized the cell fates by determining the expression of several pluripotency markers—*Pou5f1* (*Oct4*), *Nanog*, and *Rex1*. Consistent with the cell morphology and AP staining results, the expression of these markers, especially of *Nanog* and *Rex1*, were significantly decreased upon BMP4 withdrawal, and either PD184352 or SU5402 could substitute BMP4 to sustain their expression (Figure 5B). The inert response of *Oct4* to BMP4 withdrawal may be attributed to the strong differentiation-inhibitory effect of LIF, as evidenced by that *Oct4* decreased significantly when both BMP4 and LIF were deprived (Figure 5B). Thus, ERK inhibition could substitute BMP4 to maintain mouse ESC self-renewal in combination with LIF.

We then tested whether DUSP9 overexpression can mimic BMP signaling to sustain mouse ESC self-renewal. Forced expression of DUSP9 could indeed replace BMP4 in maintaining self-renewal, as demonstrated by positive AP staining and expression of NANOG and *Rex1* (Figures 5C and 5D). We also employed a lentiviral system to stably express DUSP9 in R1 ESCs with GFP as a control. Similar to transient transfection, stably expressed DUSP9 could maintain ERK activity at lower level compared to GFP control and substitute BMP4 to sustain self-renewal together with LIF as evidenced by self-renewal marker expression, AP staining, and colony formation assay (Figures S5C–S5F). Conversely, DUSP9 knockdown by siRNA oligos decreased self-renewal marker gene expression under BMP4+LIF condition (Figure 5E), indicating the requirement of DUSP9 for the self-renewal maintenance downstream of BMP4. Consistently, stable expression of dn-DUSP9 significantly compromised mouse ESC self-renewal in terms of self-renewal maker expression and cell morphology (Figures 5F, S5G, and S5H). Thus, DUSP9 is an essential mediator of BMP signaling to sustain mouse ESC self-renewal in collaboration with LIF. It has been showed that simultaneous inhibition of ERK and GSK3 activity by small molecule inhibitors PD0325901 and CHIR99021 (2i condition) can sustain ESC self-renewal (Ying et al., 2008). In the 2i condition or standard knockout serum replacement (KSR) containing ESC culture medium, however, DUSP9 knockdown had little effect on self-renewal (Figures S5I and S5J), indicating that DUSP9 is specific to mediate BMP-supported self-renewal function.

DUSP9 Contributes to BMP Inhibition of Early Neural Differentiation

When deprived of both LIF and BMP4, mouse ESCs automatically undergo early neural differentiation, and BMP signaling can repress this neural differentiation (Fei et al., 2010a; Ying et al., 2003b). Thus, we employed this system to further assess the significance of the BMP/DUSP9/ERK axis in mouse ESC fate determination.

Consistent with previous reports, BMP4 effectively inhibited the expression of neural progenitor markers—*Sox1*, *Nes* (nestin), and *Sox3* (Figure 6A). Inhibition of ERK activity by PD184352 or SU5402 yielded similar effects (Figures 6A, S6A, and S6B). These data suggest that BMP blockage of early neural differentiation may be partially through ERK inhibition. Indeed, DUSP9 overexpression led to efficient repression of the early neural markers (Figures 6B and 6C). In accordance, when dn-DUSP9 was introduced to block endogenous DUSP9 activity, the extent of BMP inhibition on the expression of *Sox1*, nestin, and *Sox3* were alleviated significantly (Figure 6D). DUSP9 knockdown by siRNA also impaired BMP4-mediated repression of neural differentiation as evidenced by neural marker gene expression and cell morphology (Figures 6E, S6C, and S6D). These results together suggest that DUSP9 contributes to the function of BMP in repression of early neural differentiation.

DISCUSSION

Extrinsic BMP and LIF signals cooperate to sustain mouse ESC self-renewal while intrinsic ERK activity primes for differentiation. How these two cytokines interact with each other to regulate self-renewal has not been clearly addressed. LIF is widely regarded as a major self-renewal regulator by activating STAT3 to inhibit differentiation while paradoxically LIF through its receptor gp130 can also activate ERK (Burdon et al., 1999; Niwa et al., 1998). BMP signaling was previously identified as a critical LIF collaborator to maintain self-renewal by upregulating ID family proteins and thus inhibiting neural differentiation (Ying et al., 2003a). Our recent study extended this notion by showing that BMP/Smad signaling promotes self-renewal also via suppression of developmental regulators' expression (Fei et al., 2010a). In the present study, we found that BMP can inhibit ERK activity through induction of the ERK-specific phosphatase DUSP9.

ERK has been documented to be a key ESC fate regulator (Kunath et al., 2007; Stavridis et al., 2007; Yoshida-Koide et al., 2004). Appropriate ERK activity is important for compromising the pluripotency and limiting ESC proliferation potential, with

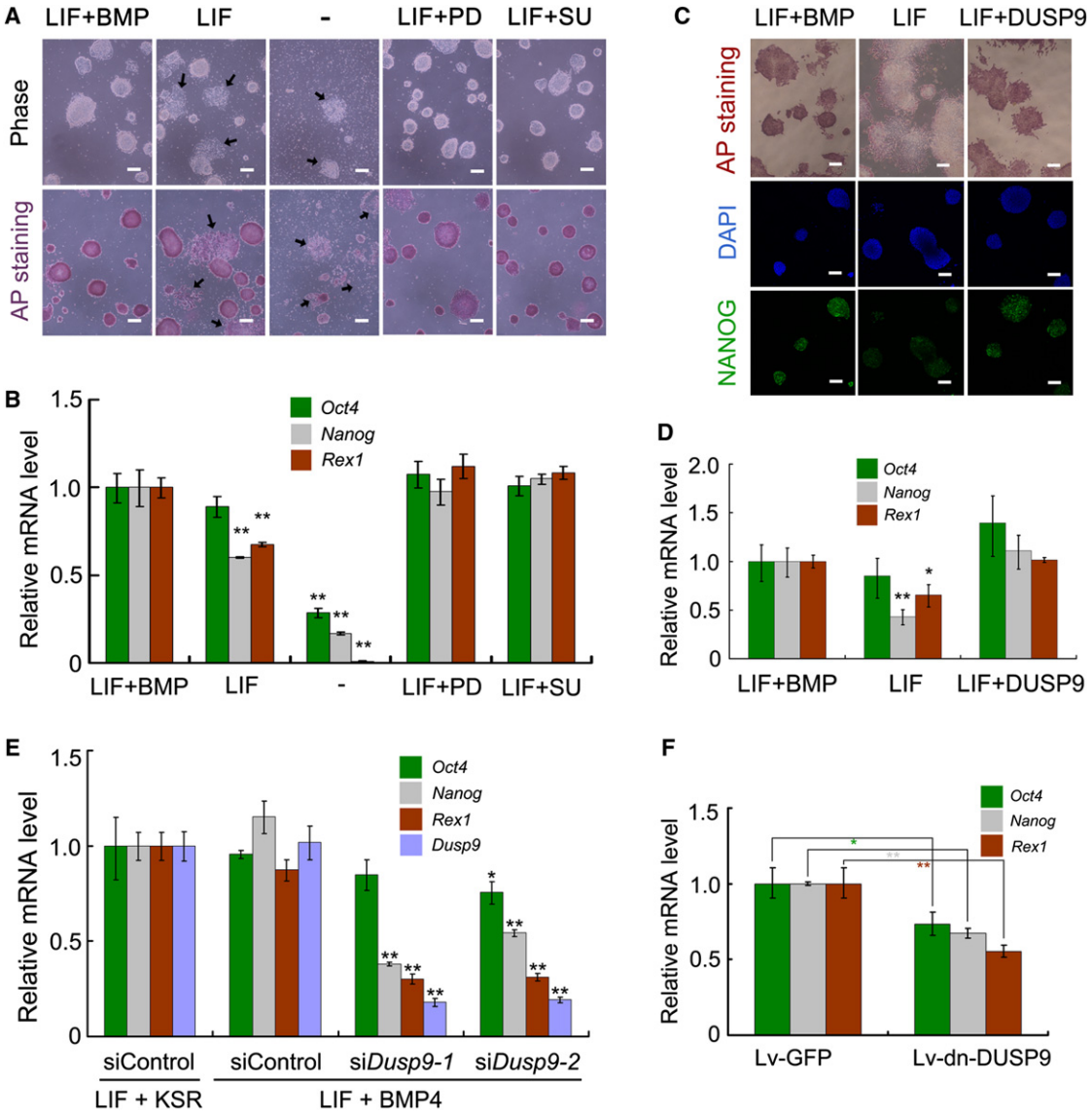

Figure 5. DUSP9 Mediates BMP4 Function to Sustain Mouse ESC Self-Renewal in the Presence of LIF

(A) Inhibition of ERK activity can substitute BMP4 in maintaining ESC self-renewal in the presence of LIF. R1 cells were cultured in N2B27 medium supplemented with LIF (10 ng/ml), BMP4 (10 ng/ml), PD184352 (1 μM), or SU5402 (2 μM) as indicated for 5 days and subjected to alkaline phosphatase (AP) staining. Arrows indicated the differentiated cells.

(B) In the presence of LIF, inhibition of ERK activity can substitute BMP4 in maintaining pluripotency marker gene expression. R1 cells were cultured in the conditions as described in (A) before being harvested for qRT-PCR.

(C) DUSP9 overexpression can substitute BMP4 in sustaining ESC self-renewal in collaboration with LIF. R1 cells were transfected with DUSP9 construct and cultured in N2B27 medium supplemented with LIF and/or BMP4 as indicated for 5 days before being subjected to AP staining or immunostaining.

(D) In the presence of LIF, DUSP9 overexpression can substitute BMP4 in maintaining pluripotency marker gene expression. R1 cells were cultured in the conditions as in (C) before being harvested for qRT-PCR.

(E) Knockdown of *Dusp9* impairs self-renewal marker gene expression in mouse ESCs cultured in LIF+BMP4 condition. R1 cells cultured in LIF+KSR or LIF+BMP4 conditions were transfected with siRNA as indicated and cells were harvested for qRT-PCR after 5 days.

(F) Ectopic expression of dn-DUSP9 reduces self-renewal marker gene expression in mouse ESCs cultured in LIF+BMP4 condition. R1 cells stably expressing GFP as a control (Lv-GFP) or expressing dn-DUSP9 (Lv-dn-DUSP9) were cultured in LIF+BMP4 condition for 6 days and then harvested for qRT-PCR.

Scale bars in (A) and (C) represent 100 μm. Data in (B) and (D)–(F) are shown as mean ± SEM (n = 3) (**p < 0.01). See also Figure S5.

higher activity to promote differentiation and lower activity to restrict it. Both exogenously added LIF and autocrine FGF signaling can activate ERK. However, the signaling to negatively regulate ERK activity remains unclear. We found that BMP can counteract the stimulating effect of LIF and FGF signaling on ERK activity, thereby maintaining mouse ESCs in the self-renewal state with an appropriate ERK activity in the presence of both LIF and BMP4. Mechanistically, the inhibitory effect of

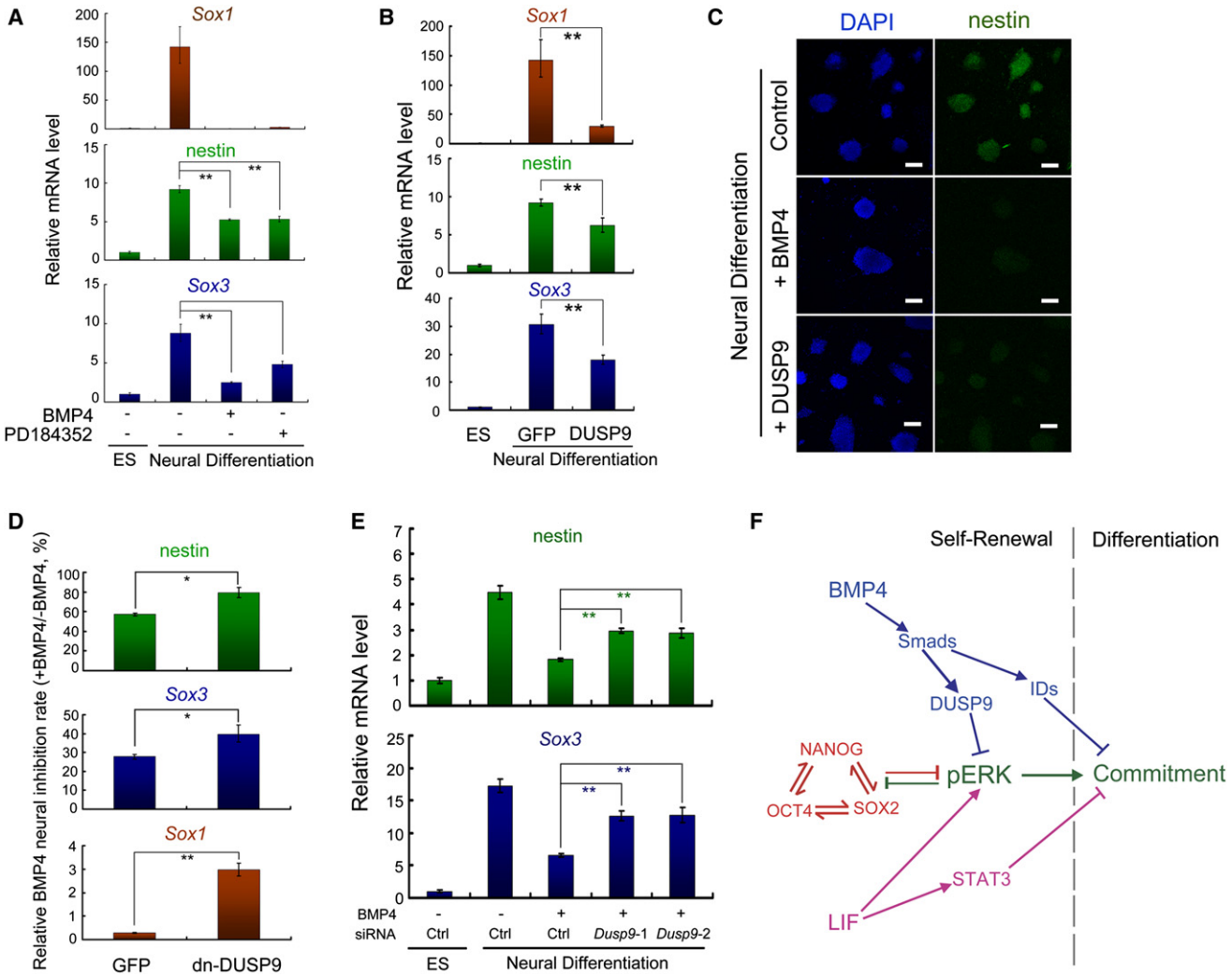

Figure 6. DUSP9 Contributes to BMP-Mediated Inhibition of Early Neural Differentiation of mESCs

(A) Inhibition of ERK activity by PD184352 mimics the inhibitory effect of BMP4 on early neural differentiation. R1 cells cultured in neural differentiation condition (N2B27 medium without growth factors) supplemented with or without 10 ng/ml BMP4 or 1 μM PD184352 for 5 days before being harvested for qRT-PCR.

(B) DUSP9 overexpression mimics the inhibitory effect of BMP4 on early neural differentiation. R1 cells transfected with plasmids expressing DUSP9 or GFP were cultured in N2B27 medium for 5 days before being harvested for qRT-PCR.

(C) DUSP9 overexpression mimics BMP4 in inhibiting nestin expression. R1 cells transfected with or without DUSP9 were cultured in N2B27 medium with or without BMP4 for 5 days and subjected to anti-nestin immunostaining. Scale bars represent 100 μm.

(D) dn-DUSP9 attenuates the inhibitory effect of BMP on neural differentiation. R1 cells transfected with dn-DUSP9 or GFP construct were cultured in N2B27 medium with or without BMP4 for 5 days and then harvested for qRT-PCR. BMP4 neural inhibition rate (+BMP4/−BMP4) was calculated by division of the mRNA levels in the presence of BMP4 to the ones in the absence of BMP4 (n = 3) (*$p < 0.05$; **$p < 0.01$).

(E) Knockdown of *Dusp9* impairs the inhibitory effect of BMP4 on early neural differentiation of ESCs. R1 cells cultured in standard KSR-containing ESC medium or N2B27 medium with or without BMP4 were transfected with siRNA as indicated. Cells were harvested for qRT-PCR after 5 days.

(F) Schematic model for the function of DUSP9 in mediating BMP signaling to control ERK activity during mouse ESC fate determination.

Data in (A), (B), (D), and (E) are shown as mean ± SEM (n = 3) (**$p < 0.01$). See also Figure S6.

BMP on ERK is via Smad-dependent upregulation of DUSP9. Thus, by identification of DUSP9 as an important BMP target, we establish the link between BMP signaling and regulation of ERK activity during mouse ESC fate determination.

Several previous studies have suggested the link between BMP signaling and MAPK activities (Qi et al., 2004; Ying et al., 2003a, 2008; Zhang et al., 2010). Ying et al. (2003a) reported that BMP4 can slightly activate ERK activity within a short time period (15 min and 1 hr), which is consistent with our data. It has been shown that BMP signaling may support mouse ESC self-renewal by inhibition of MAPK pathways, in which ERK activity was only transiently repressed in 5 min and then completely recovered at 1 hr, whereas p38 seemed more sensitive to BMP inhibition within 1 hr (Qi et al., 2004). A recent report also indicated that BMP signaling can inhibit ERK phosphorylation within 10 min (Zhang et al., 2010). Despite these indications,

the exact link between BMP signaling and ERK activity has not been firmly established. We carefully monitored the BMP effects on ERK activity in a long time course and found that BMP slightly activates ERK within 4 hr but causes a steady inhibition of ERK after 8 hr and beyond. Because BMP is constantly included in the LIF+BMP protocol to culture mouse ESCs, our results should reflect the most actual effect of BMP on ERK activity in mouse ESCs.

The slow responsiveness of ERK to BMP4 treatment led us to speculate that BMP signaling represses ERK activation through gene transcription. Consistent with this notion, de novo protein synthesis was required for the inhibitory effect of BMP on ERK activity, and the ERK-specific phosphatase DUSP9 was among the potential targets of BMP/Smad signaling (Fei et al., 2010a). DUSPs negatively modulate MAPK activities by dephosphorylating serine/threonine and tyrosine residues. Different DUSPs have distinct expression pattern, substrate selectivity, and biological functions (Jeffrey et al., 2007; Patterson et al., 2009; Theodosiou and Ashworth, 2002). Our data showed that only DUSP9 exhibits specific upregulation by BMP signaling in mouse ESCs, and more importantly, inhibition of endogenous DUSP9 activity efficiently blocks the BMP suppression of ERK activity, placing DUSP9 as a crucial mediator of BMP signaling to interfere with ERK activity. Further detailed analysis firmly established that Smad1/5 and Smad4 are responsible for BMP upregulation of *Dusp9*.

We have also demonstrated that DUSP9 can mediate BMP function in mouse ESC fate determination through inhibition of ERK. First, by employing the BMP4+LIF self-renewal maintenance system, we found that inhibition of ERK activity by DUSP9 overexpression, like the MEK1 inhibitor PD184352 or the FGF receptor inhibitor SU5402, can substitute BMP4 to support self-renewal in the presence of LIF. Loss-of-function experiments by dn-DUSP9 and DUSP9 knockdown also indicated that DUSP9 is necessary for BMP-sustained self-renewal in cooperation with LIF. Second, in an early neural differentiation system without LIF, BMP treatment and loss-of-function of ERK attenuated early neural differentiation, as reported previously (Fei et al., 2010a; Kunath et al., 2007; Stavridis et al., 2007; Ying et al., 2003b). Overexpression of DUSP9 could interfere with this differentiation process. Furthermore, the decrease of early neural markers was significantly alleviated by dn-DUSP9 and DUSP9 knockdown, indicating the important role of DUSP9 in mediating BMP inhibition of early neural differentiation. ID proteins can partially substitute BMP4 to inhibit neural differentiation (Zhang et al., 2010), and suppression of differentiation genes also contributes to BMP promotion of self-renewal (Fei et al., 2010a). Thus, multiple targets may work together to mediate BMP function to promote self-renewal and inhibit differentiation of mouse ESCs.

We also tried to extend our findings to human ESCs. Consistent with that in mouse ESCs, BMP4 can upregulate *Dusp9* expression in the protein level (Figure S6E) as well as in the mRNA level in both H1 hESCs (Figure S6F) and H9 hESCs (Figure S6G). Similarly, BMP4 can still inhibit ERK phosphorylation in H1 human ESCs (Figure S6E). Thus, the BMP-ERK connection and BMP4 upregulation of DUSP9 are conserved in human ESCs. However, the functional output of BMP signaling and ERK signaling were in great contrast between human and mouse ESCs (Bernardo et al., 2011; Li et al., 2007; Xu et al., 2002; Ying et al., 2003a, 2008). Further efforts are required to clarify whether DUSP9 functionally connects BMP signaling and ERK pathway in human ESC fate determination.

The correlated expression pattern of Smad4 and DUSP9 during early embryonic development suggested certain physiological significance of the BMP/Smad/DUSP9 axis. *Dusp9*$^{-/-}$ mice showed lethality around E10.5 resulting from defective extraembryonic tissue development (Christie et al., 2005), which is similar to that of *Erk2*$^{-/-}$ mice (Hatano et al., 2003). Interestingly, although *Smad4*$^{-/-}$ mice die at around E6.5–E8.5, earlier than *Dusp9*$^{-/-}$ mice, they also exhibit disordered extraembryonic tissue development (Sirard et al., 1998; Yang et al., 1998). These genetic studies strongly suggested that the BMP/Smad/DUSP9 cascade and ERK are interconnected to regulate embryonic development.

In summary, our present study firmly establishes that DUSP9, as a critical direct target of BMP/Smad signaling, plays a critical role in mediating BMP functions during mouse ESC fate decisions by inhibition of ERK activity. The elucidation of how extrinsic BMP signaling interacts with the critical intrinsic cell fate calibrator ERK provides insight into a better understanding of delicate ESC fate determination.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Antibodies

R1 and E14 mouse ESCs were maintained as described previously (Fei et al., 2010b). For serum- and feeder-free culture, cells were grown on gelatinized tissue culture plates in N2B27 medium (Ying et al., 2003a) with LIF (Chemicon) and/or BMP4 (R&D Systems) in concentration of 10 ng/ml, unless specified in the figure legends. For early neural differentiation, R1 cells were cultured in N2B27 medium without any other cytokines as described previously (Fei et al., 2010a). The 2i culture condition for mouse ESCs, the culture conditions for mouse somatic cell lines, H1 and H9 human ESC lines, and reagents and antibodies are described in Supplemental Information. Mice manipulations followed the protocols approved by the Tsinghua Animal Ethics Committee.

Lentiviral Transduction

The coding sequences (CDS) of GFP, GFP-DUSP9, or GFP-dn-DUSP9 were cloned into pENTR1A plasmid and LR clonase reactions (Invitrogen) were carried out to place these CDS under the control of EF1α promoter in the p2k7$_{neo}$ lentiviral backbone (gift from Dr. Kehkooi Kee) (Kee et al., 2009). The viral supernatants were used to infect mouse ESCs. Geneticin (250 μg/ml, Invitrogen) was added into culture medium for 5 days to select cells with stable viral integration.

Knockdown by siRNA

siRNA were purchased from GenePharma (Shanghai). siRNA (100–200 nM) were transfected into mouse ESCs with DharmaFECT1 (Dharmacon) according to the manufacturer's instruction. siRNAs were transfected every 2 days in long-term functional assays to ensure efficient knockdown of target genes. siRNA sequences were listed in the Supplemental Information.

Immunoblotting, Immunostaining, Luciferase Reporter Assay, RNA Isolation, Reverse Transcription, and qRT-PCR

Tests performed as described previously (Fei et al., 2010a). Detailed protocol described in Supplemental Information.

Secondary Colony Formation Assay and Alkaline Phosphatase Staining

Secondary colony formation assay was performed as previously described (Yuan et al., 2009). Alkaline phosphatase staining was performed according

to the manufacturer's instructions (SCR004, Millipore). Detailed protocol described in Supplemental Information.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was carried out as described previously (Fei et al., 2010a). Detailed protocol and specific PCR primers for ChIP are described in Supplemental Information.

Statistic Analysis

All the values were shown as mean ± SEM. The significance between groups was determined by Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.stem.2011.12.016.

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