Brief UltraRapid Communication

Jmjd3 Controls Mesodermal and Cardiovascular Differentiation of Embryonic Stem Cells

Kisho Ohtani,* Cong Zhao,* Gergana Dobreva, Yosif Manavski, Britta Kluge, Thomas Braun, Michael A. Rieger, Andreas M. Zeiher, Stefanie Dimmeler

Rationale: **The developmental role of the H3K27 demethylases Jmjd3, especially its epigenetic regulation at target genes in response to upstream developmental signaling, is unclear.**

Objective: **To determine the role of Jmjd3 during mesoderm and cardiovascular lineage commitment.**

Methods and Results: **Ablation of Jmjd3 in mouse embryonic stem cells does not affect the maintenance of pluripotency and self-renewal but compromised mesoderm and subsequent endothelial and cardiac differentiation. Jmjd3 reduces H3K27me3 marks at the *Brachyury* promoter and facilitates the recruitment of β-catenin, which is critical for Wnt signal–induced mesoderm differentiation.**

Conclusions: **These data demonstrate that Jmjd3 is required for mesoderm differentiation and cardiovascular lineage commitment. (*Circ Res*. 2013;113:856-862.)**

Key Words: Brachyury protein ■ embryonic stem cells ■ epigenomics ■ Jmjd3 protein, mouse ■ mesoderm ■ Wnt signaling pathway

Post-translational modifications of histone proteins represent essential epigenetic control mechanisms that can either allow or repress gene expression.[1] Trimethylation of H3K27 is mediated by Polycomb group proteins and represses gene expression.[2] The JmjC domain–containing proteins, UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome) and Jmjd3 (jumonji domain–containing protein 3, Kdm6b), not only act as demethylases to remove the repressive H3K27me3 marks, but also exhibit additional demethylase-independent functions.[3–6] Jmjd3 is induced and participates in *Hox* gene expression during development,[7] neuronal differentiation,[8,9] and inflammation,[5,10–12] and recent data suggest that Jmjd3 inhibits reprogramming by inducing cellular senescence.[13] Because previous studies suggest that H3K27me3 regulates endothelial gene expression in adult proangiogenic cells,[14] we addressed the function of Jmjd3 in cardiovascular lineage differentiation of embryonic stem cells (ESCs).

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Methods

A detailed description of the experimental procedure is provided in the Online Data Supplement.

Cell Culture

Mouse ESCs were cultured and maintained on mitotically inactivated mouse embryonic fibroblasts. Differentiation of ESCs was induced without leukemia inhibitory factor in static or rotary suspension condition. ESC differentiation into mesoderm was performed with a well-described OP9 coculture system.[15,16] For cardiac differentiation, embryoid bodies (EBs) were derived using the hanging drop method.[17] For endothelial differentiation, EBs were formed and cultured in medium supplemented with cytokine cocktail.[18]

Plasmid Construction and Stable Transfection

The full-length *Jmjd3*, the mutants, and *Brachyury* were cloned into pEF1 vector (Invitrogen). The linearized plasmids were transfected in *Jmjd3*$^{-/-}$ ESCs using the Amaxa nucleofection system (Lonza).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed as previously described.[14] Sheared chromatin was immunoprecipitated with antibodies as indicated. The purified chromatin was used in quantitative real-time polymerase chain reaction (Applied Biosystems).

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From the Institute of Cardiovascular Regeneration, Centre for Molecular Medicine, University of Frankfurt, Frankfurt, Germany (K.O., C.Z., Y.M., B.K., S.D.); Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany (G.D., T.B.); Department of Hematology/Oncology, Internal Medicine II, LOEWE Center for Cell and Gene Therapy, University of Frankfurt, Frankfurt, Germany (M.A.R.); and Department of Cardiology, Internal Medicine III, University of Frankfurt, Frankfurt, Germany (A.M.Z.).

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*These authors contributed equally to this article.

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Correspondence to Stefanie Dimmeler, Institute of Cardiovascular Regeneration, Centre for Molecular Medicine, University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. E-mail dimmeler@em.uni-frankfurt.de

Nonstandard Abbreviations and Acronyms	
EB	embryoid body
ESC	embryonic stem cell
WT	wild type

Results

Jmjd3 knockout ESCs were generated by 2 rounds of gene targeting (Online Figure IA and IB). We obtained 7 *Jmjd3*$^{-/-}$ ESC clones, which lacked *Jmjd3* mRNA and protein expression. All of the clones showed slightly increased global H3K27me3, but the expression of pluripotency genes, the morphology, the growth kinetic, and survival was indistinguishable from wild-type (WT) ESCs (Figure 1A–1C; Online Figure IC–IF). No significant changes of repressive H3K27me3 marks at the promoters of pluripotency genes were detected in *Jmjd3*$^{-/-}$ compared with WT ESCs (Online Figure IH). When spontaneous differentiation was induced by leukemia inhibitory factor withdrawal, *Jmjd3* expression increased in WT ESCs (Figure 1D). EBs derived from *Jmjd3*$^{-/-}$ ESCs were slightly smaller in size compared with WT EBs (Figure 1E). mRNA expression profiling of *Jmjd3*$^{-/-}$ and WT ESC clones at day 4 after induction of differentiation showed a distinct expression pattern of lineage-specific genes (Online Figure IIA). Gene ontology functional analyses revealed a significant repression of genes that are involved in mesoderm development (Figure 1F; Online Figure IIB). Moreover, repressed gene sets in *Jmjd3*$^{-/-}$ EBs were shown to be related to cardiac and vascular development, consistent with impaired mesoderm differentiation (Figure 1F; Online Figure IIB).

Validation of the microarray results showed a similar reduction of pluripotency gene expression after leukemia inhibitory factor withdrawal in *Jmjd3*$^{-/-}$ compared with WT ESCs (Figure 1G). However, depletion of Jmjd3 substantially compromised the induction of mesodermal genes (Figure 1G). Especially, the pan-mesoderm marker, *Brachyury*, and the early mesoendoderm marker, *Mixl1*, were profoundly increased at day 4 of differentiation in WT ESCs, but not in Jmjd3$^{-/-}$ ESCs (Figure 1G). Moreover, the mesoendodermal marker, *Eomes*, and endodermal markers, such as *Sox17* and *FoxA2*, were significantly suppressed, which is consistent with a very recent study showing that Jmjd3 is required for endoderm differentiation.[19] Ectodermal markers were not significantly changed in *Jmjd3*$^{-/-}$ ESCs when using the spontaneous differentiation protocol (Figure 1G).

Because *Jmjd3*$^{-/-}$ ESCs showed a prominent inhibition of mesodermal markers after leukemia inhibitory factor withdrawal, we next questioned whether this phenotype can also be observed when directing differentiation of mesoderm using 2 different protocols. Consistent with our findings, *Jmjd3*$^{-/-}$ ESCs showed a reduced expression of mesodermal marker genes when using the protocol for mesoderm differentiation described by Gadue et al[20] (data not shown). Moreover, mesoderm differentiation was significantly suppressed when *Jmjd3*$^{-/-}$ ESCs were cultured on OP9 stromal cells, which support mesodermal differentiation[21] (Figure 2A). Whereas WT ESCs showed the typical time-dependent increase in Brachyury^{+} cells, *Jmjd3*$^{-/-}$ ESCs generated significantly less Brachyury^{+} mesodermal cells (Figure 2B). Moreover, fluorescence activated cell sorting analysis revealed that fetal liver kinase (Flk)1^{+} vascular endothelial-cadherin^{-} mesodermal cells were generated in WT ESCs but were reduced when *Jmjd3*$^{-/-}$ ESCs were used (Figure 2C). Interestingly, the formation of vascular endothelial-cadherin^{+} Flk^{+} cells was also significantly reduced by 96±1% and 88±3% in the 2 *Jmjd3*$^{-/-}$ ESC clones compared with WT ESCs (P<0.01), prompting us to explore the role of Jmjd3 in vascular differentiation further.

Endothelial differentiation was induced by a cytokine cocktail[18] and was associated with a significant upregulation of *Jmjd3* expression (Online Figure IIIA). *Jmjd3*$^{-/-}$ ESCs showed a marked reduction of endothelial differentiation as evidenced by significantly reduced mRNA levels of the endothelial marker vascular endothelial-cadherin and endothelial-specific receptor tyrosine kinase *Tie2* (Figure 3A). The formation of endothelial marker expressing vascular structures after induction of endothelial differentiation was abolished in *Jmjd3*$^{-/-}$ ESCs (Figure 3B; Online Figure IIIB). The impaired endothelial differentiation of Jmjd3$^{-/-}$ cells was partially rescued by the overexpression of *Brachyury* (Online Figure IIIC and IIID), suggesting that the inhibition of mesoderm formation, at least in part, contributes to the impaired endothelial commitment.

Because genes involved in heart development and morphogenesis were significantly downregulated in *Jmjd3*$^{-/-}$ ESCs on differentiation (Figure 1F; Online Figure II), we additionally determined the capacity of Jmjd3$^{-/-}$ ESCs to generate cardiomyocytes by inducing cardiac differentiation.[17] Expression of cardiac progenitor cell markers, *Mesp1* and *Pdgfra*, was inhibited in *Jmjd3*$^{-/-}$ ESCs compared with WT ESCs (Figure 3C). Moreover, after plating on gelatin-coated dishes, the Jmjd3$^{-/-}$ ESCs showed an impaired formation of EBs and only 20% of EBs were contracting (Figure 3D). Consistently, expression of the cardiac transcription factor *Mef2c*, the marker of working myocardium *Nppa*, and cardiac structural proteins *TnT2* and α-myosin heavy chain were downregulated in *Jmjd3*$^{-/-}$ ESCs (Figure 3E and 3F; Online Figure IIIE).

Next, we addressed whether the impaired mesoderm differentiation observed in *Jmjd3*$^{-/-}$ ESCs might be mediated by an increase of repressive H3K27me3 marks at the promoters of developmental regulators. Of the various promoters studied, only *Brachyury* and *Mixl1* showed a significant augmentation of H3K27me3 marks in *Jmjd3*$^{-/-}$ ESCs on differentiation (Figure 4A; Online Figure IVA). Consistently, the recruitment of RNA polymerase II to the transcription start sites of the promoters of *Brachyury* and *Mixl1* was also significantly reduced (Online Figure IVC). In addition, Jmjd3 deficiency repressed polymerase II recruitment to the *Flk1* and *Mesp1* promoter but the inactivation of these promoters was not associated with changes in H3K27me3 marks (Figure IVA and IVC). These data were confirmed using protocols that induce mesoderm differentiation by addition of Wnt3a (data not shown).[20] Under these conditions, *Jmjd3*$^{-/-}$ ESCs showed a 1.81±0.23-fold (P<0.05) enrichment of H3K27me3 marks at the *Brachyury* promoter compared with WT ESCs.

To determine whether the demethylase activity of Jmjd3 controls *Brachyury* expression by reducing repressive H3K27me3 marks during differentiation, we overexpressed full-length Jmjd3, the carboxyl-terminal part, including the JmjC-domain (cJmjd3:

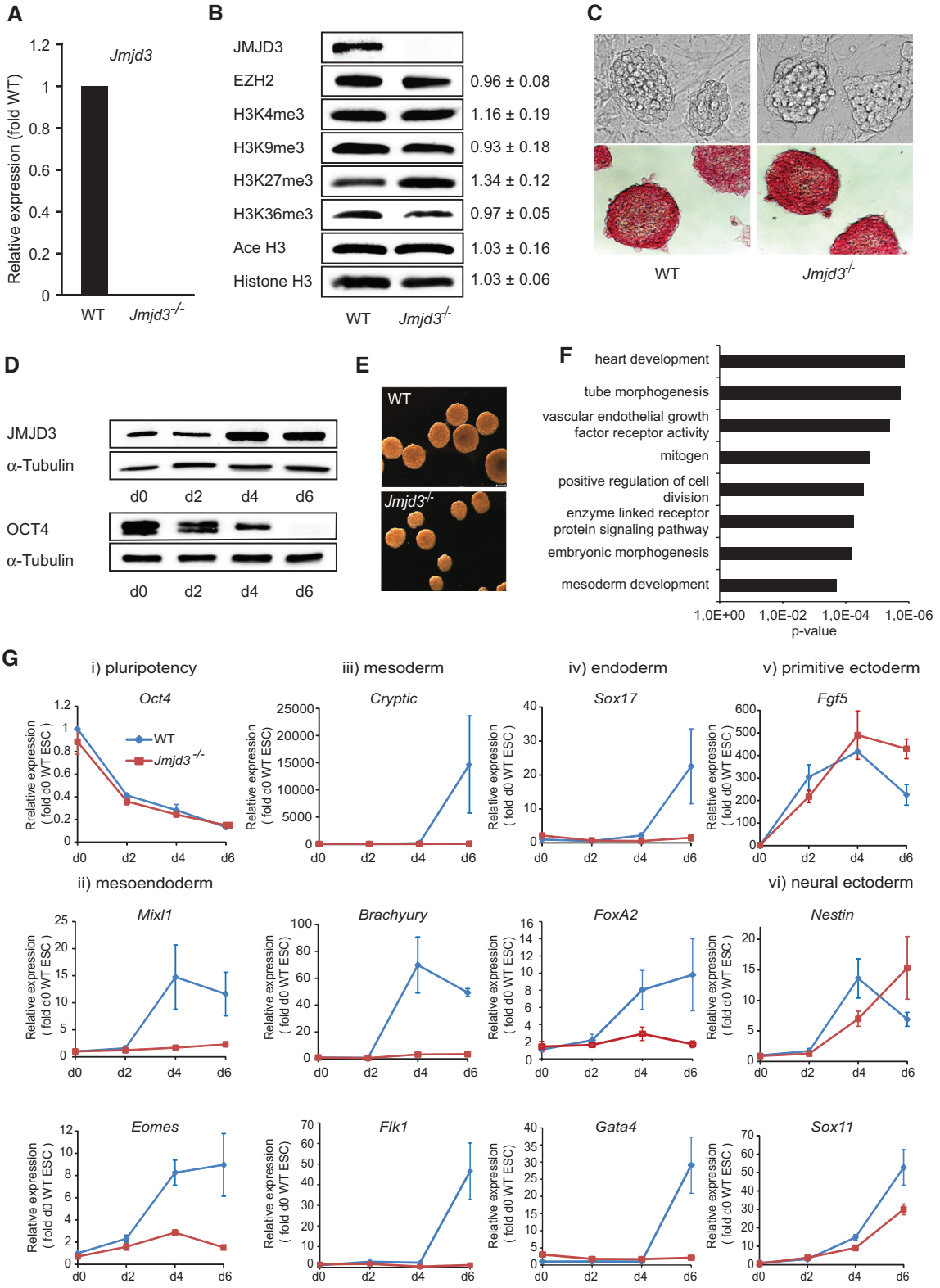

Figure 1. Aberrant differentiation of *Jmjd3*$^{-/-}$ embryonic stem cells (ESCs). **A**, Quantitative polymerase chain reaction analysis of *Jmjd3* in wild-type (WT) and *Jmjd3*$^{-/-}$ ESCs. **B**, Western blot analysis of *Jmjd3* and Histone marks in WT and *Jmjd3*$^{-/-}$ ESCs. Histone H3 is used as a loading control. Quantification is shown in the **right** (n=3–5). **C**, **Top**, Morphology of WT and *Jmjd3*$^{-/-}$ ESCs on feeder cells. **Bottom**, Alkaline phosphatase staining of undifferentiated WT and *Jmjd3*$^{-/-}$ ESCs. **D**, Western blot analysis of Jmjd3 and Oct4 in WT ESCs during differentiation. α-Tubulin is used as a loading control. **E**, Bright field image of embryoid bodies at day 5. Scale bar, 200 μm. **F**, Gene ontology analysis for >2-fold repressed genes in *Jmjd3*$^{-/-}$ ESCs compared with WT ESCs 4 days after differentiation. The most highly represented categories are presented with ontology terms on the *y*-axis and *P* values for the significance of enrichment are shown on the *x*-axis. **G**, Gene expression changes of pluripotency and lineage-specific markers in WT and *Jmjd3*$^{-/-}$ ESCs after spontaneous differentiation by leukemia inhibitory factor withdrawal (n=4). Flk indicates fetal liver kinase.

amino acids, 1141–1641), and a carboxyl-terminal mutant construct, which includes a point mutation (cJmjd3H1388A) to inactivate demethylase activity. Overexpression of full-length Jmjd3 and the carboxyl-terminal part of Jmjd3 in *Jmjd3*$^{-/-}$ ESCs partially rescued the expression of *Brachyury* on differentiation (Figure 4B and 4C). However, the inactive carboxyl-terminal part of Jmjd3

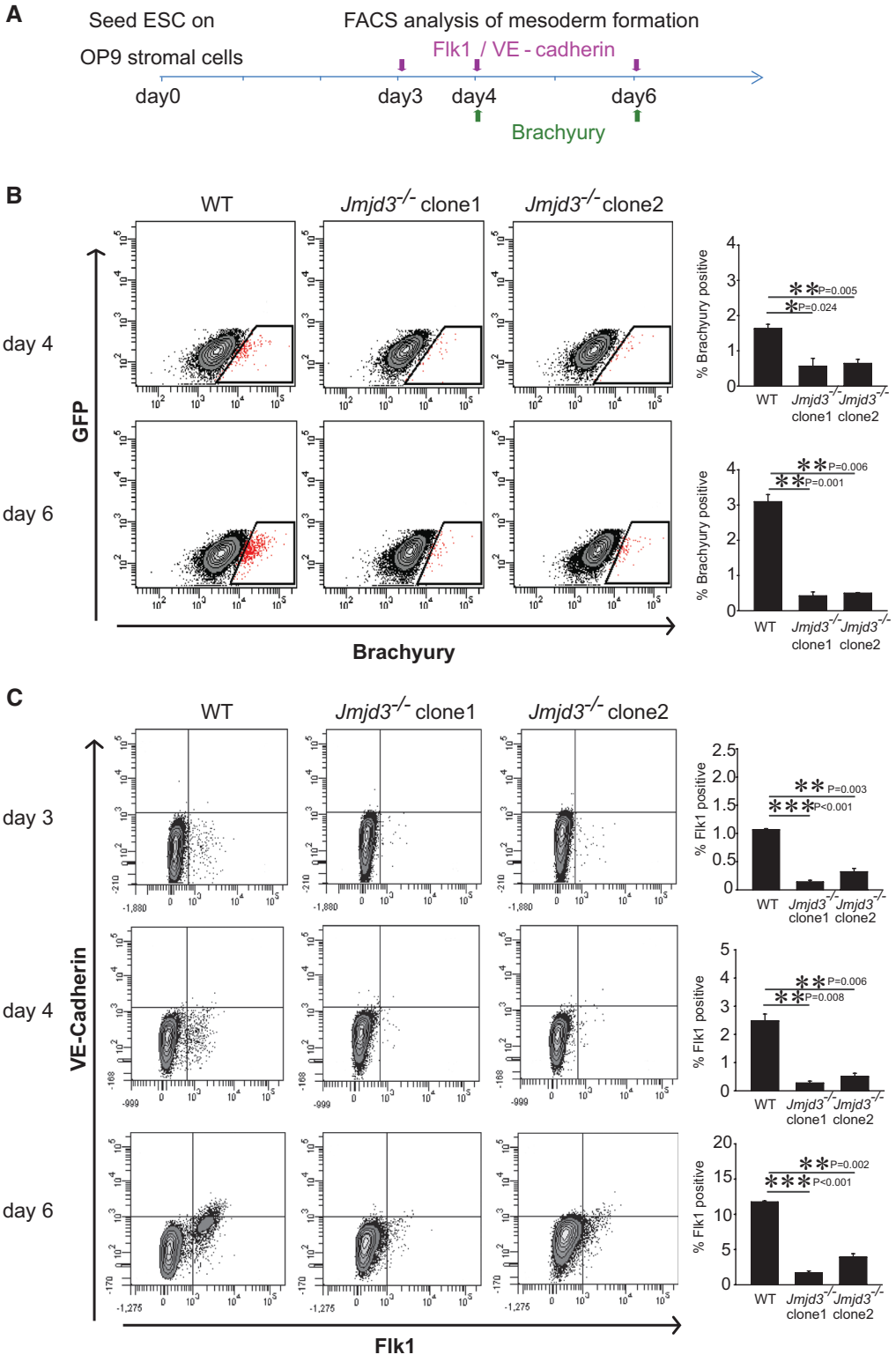

Figure 2. *Jmjd3*$^{-/-}$ embryonic stem cells (ESCs) show an impaired ability to differentiate into mesoderm. A, Schematic illustration of the experimental protocol. Differentiation of ESCs (wild-type [WT] and 2 *Jmjd3*$^{-/-}$ ESCs clones) on OP9 feeder cells was analyzed. **B**, **Left**, Representative fluorescence activated cell sorting (FACS) plots showing *Brachyury* expression of ESC-derived cells. **Right**, Quantification of FACS analyses (n=3). **C**, **Left**, Representative FACS plots showing fetal liver kinase 1 (Flk1) and vascular endothelial-cadherin expression on ESC-derived cells. **Right**, Quantification of FACS analyses in Flk1^{+} cells (n=3).

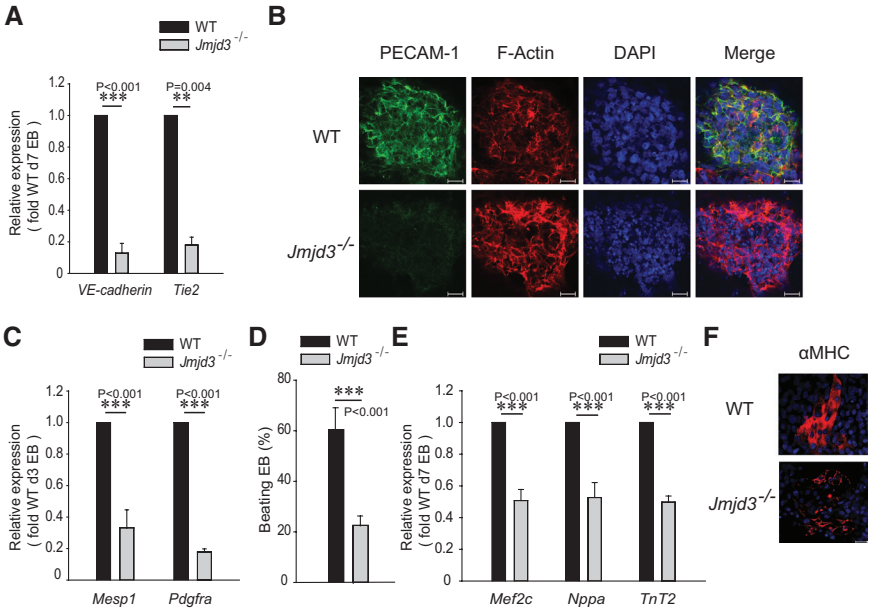

Figure 3. Jmjd3 is required for embryonic stem cells (ESCs) differentiation to the endothelial and cardiac lineage. **A**, mRNA expression of endothelial markers at day 7 of endothelial differentiation (n=3). **B**, Platelet endothelial cell adhesion molecule (Pecam)-1 staining of wild-type (WT) and *Jmjd3*$^{-/-}$ ESCs at day 8 of endothelial differentiation. Phalloidin is used to stain F-actin. Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). Scale bar, 20 μm. **C**, Gene expression of cardiac progenitor markers at day 3 of cardiac differentiation. **D**, Number of beating embryoid bodies (EBs) at day 10 of cardiac differentiation (n=8). **E**, Gene expression of cardiac markers at day 7 of cardiac differentiation (n=6). **F**, α-Myosin heavy chain staining of WT and *Jmjd3*$^{-/-}$ ESCs at day 9 of cardiac differentiation. Nuclei are stained with Hoechst (blue). Scale bar, 20 μm. **P*<0.05, ***P*<0.01, and ****P*<0.001.

failed to rescue the impaired *Brachyury* expression in Jmjd3$^{-/-}$ ESCs (Figure 4C), suggesting that the demethylase activity of Jmjd3 is required for the activation of the *Brachyury* promoter.

Because canonical Wnt signaling regulates the expression of *Brachyury* during development[22,23] and Wnt/β-catenin–dependent genes were suppressed in Jmjd3$^{-/-}$ EBs compared with WT EBs (Online Figure V), we further explored whether Jmjd3 might interact with β-catenin signaling. Indeed, β-catenin recruitment to the *Brachyury* promoter was significantly suppressed in *Jmjd3*$^{-/-}$ ESCs (Figure 4D) and was rescued by *Jmjd3* overexpression (Figure 4E). Similar results were obtained when using the protocol for direct mesoderm differentiation described by Gadue et al[20] (data not shown). To determine whether Jmjd3 might interact with β-catenin, we performed coimmunoprecipitation studies and showed that Jmjd3 interacts with β-catenin in human embryonic kidney 293 cell and differentiated EBs (Figure 4F; Online Figure VI). To assess a direct effect of Jmjd3 on β-catenin responsive promoter activity, we used a luciferase reporter assay. Coexpression of lymphoid enhancer binding factor 1 and the constitutive active form of β-catenin harboring a nuclear localization signal resulted in the activation of lymphoid enhancer binding factor 1 luciferase reporter activity in WT ESCs, but this transcriptional activation was markedly impaired in Jmjd3$^{-/-}$ ESCs (Figure 4G).

Discussion

The data of the present study demonstrate that deletion of Jmjd3 in ESCs does not affect self-renewal but significantly impairs the formation of mesoderm on induction of differentiation. The findings that Jmjd3 is not required for ESC maintenance are consistent with the dispensability of the Polycomb complex and the related demethylase UTX for self-renewal.[1] The requirement of Jmjd3 for mesoderm differentiation was shown in spontaneous differentiation, as well as when more specifically inducing mesoderm differentiation by the OP9 coculture system or under serum-free conditions followed by Wnt3a stimulation. Jmjd3 deficiency profoundly suppressed the expression of *Brachury*, which is essential for mesoderm differentiation. In the absence of Jmjd3, repressive H3K27me3 marks at the *Brachyury* promoter are significantly increased, and the recruitment of β-catenin, which is a prerequisite for Wnt-induced mesoderm differentiation, is impaired. In addition, Jmjd3 is interacting with β-catenin and is contributing to β-catenin–dependent promoter activation. This is consistent with the recent findings that cofactors can form a complex with β-catenin/lymphoid enhancer binding factor 1 at Tcf/lymphoid enhancer binding factor 1 binding sites at β-catenin–dependent promoters and synergize with canonical Wnt signaling.[24] Interestingly, a demethylase-independent regulation of β-catenin–dependent gene expression was recently described for UTX.[25] However, our data provide evidence that *Brachyury* expression in *Jmjd3*$^{-/-}$ ESCs is only rescued by catalytically active Jmjd3, which has maintained the demethylase activity. On the basis of these findings, we propose a model in which Jmjd3 is recruited to the *Brachury* promoter to remove repressive H3K27me3 marks and on Wnt stimulation additionally promotes β-catenin–dependent promoter activation (Figure 4H). Such a model is similar to the recently described function of Jmjd3 in endoderm differentiation, whereby Jmjd3 associates with Tbx3 and is recruited to the poised promoter of *Eomes*, to mediate chromatin remodeling allowing subsequent induction of endoderm differentiation induced by activin A.[19] The present study additionally demonstrates that Jmjd3 contributes to endothelial and cardiac differentiation. Particularly, endothelial differentiation was profoundly impaired, a finding that is consistent with previous findings in adult progenitor cells, showing a high H3K27me3 at endothelial genes.[14] The relatively modest inhibition of cardiomyocyte differentiation in *Jmjd3*$^{-/-}$ ESCs may be, in part, explained by a compensatory effect of UTX which was shown to regulate cardiac development.[26] Together, our study provides first evidence for the regulation of β-catenin–dependent Wnt target genes by Jmjd3 during differentiation of ESCs.

However, the in vivo relevance of the findings is still unclear. The *Jmjd3*$^{-/-}$ mice that we have generated out of the ESCs, used in the present study, showed embryonic lethality before E6.5, suggesting a crucial role of Jmjd3 in early embryonic development

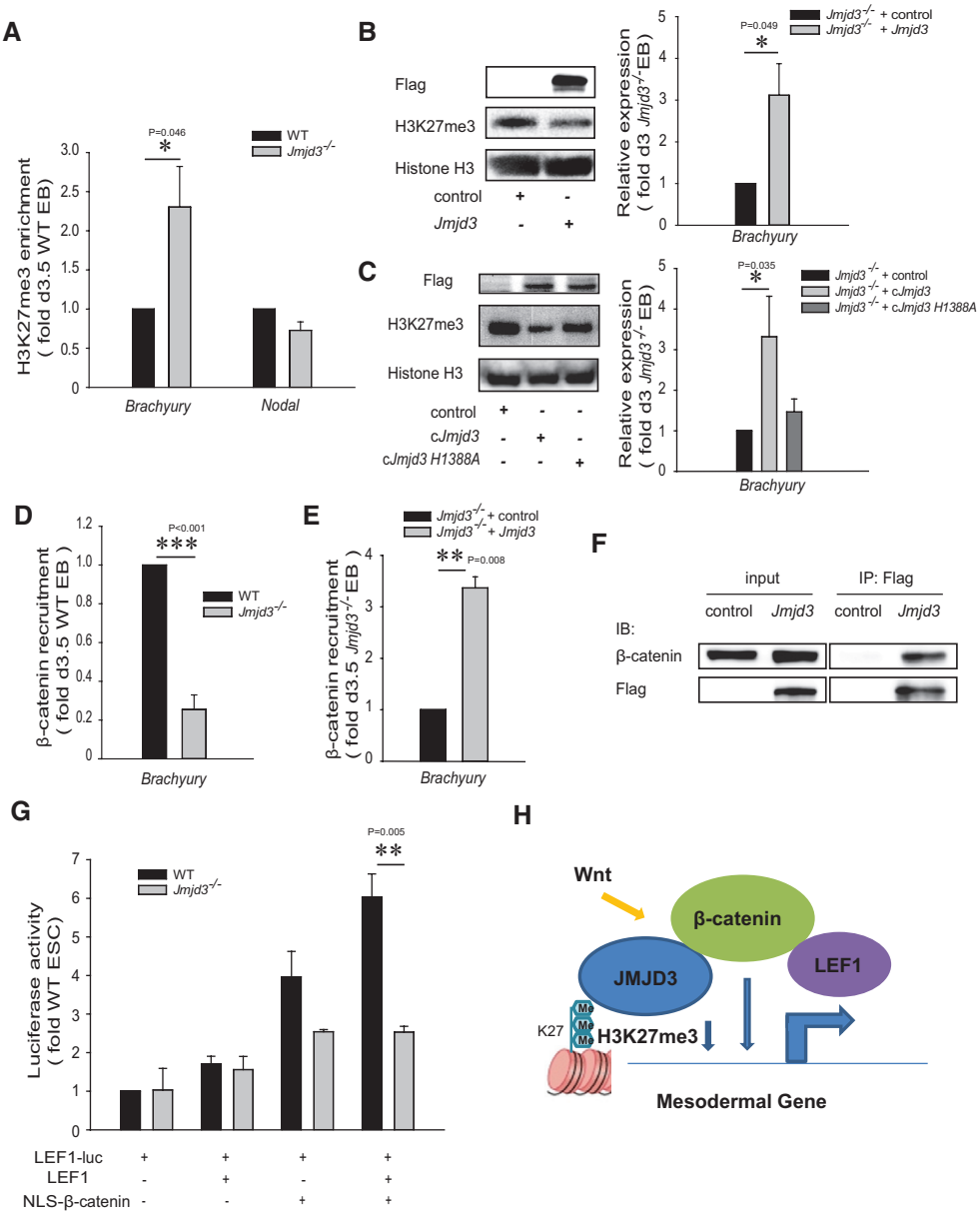

Figure 4. A, Chromatin immunoprecipitation (ChIP) against H3K27me3 at day 3.5 during differentiation of wild-type (WT) and *Jmjd3*$^{-/-}$ embryonic stem cells (ESCs). ChIP enrichments are normalized to histone H3 density and are represented as fold change relative to WT (n=4). **B**, **Left**, Western blot analysis of H3K27me3 in *Jmjd3*$^{-/-}$ ESCs after overexpression of Flag-tagged Jmjd3. **Right**, Brachyury expression at day 4 of differentiation in *Jmjd3*$^{-/-}$ ESCs after overexpression of Jmjd3 (n=3). **C**, **Left**, Western blot analysis of H3K27me3 in *Jmjd3*$^{-/-}$ ESCs after overexpression of the Flag-tagged carboxyl-terminal (C-terminal) catalytically active or inactive domain of Jmjd3. **Right,** Brachyury expression at day 4 of differentiation in *Jmjd3*$^{-/-}$ ESCs after overexpression of catalytically active or inactive Jmjd3 C-terminal domain (n=3). **D**, ChIP against β-catenin in WT and *Jmjd3*$^{-/-}$ embryoid bodies (EBs) at day 3.5 of differentiation. ChIP enrichments are calculated as percentage of input signal and data are presented as fold changes compared with WT (n=3). **E**, ChIP assay against β-catenin at day 3.5 differentiation in *Jmjd3*$^{-/-}$ EBs after overexpression of *Jmjd3*. ChIP enrichments are presented as fold changes compared with *Jmjd3*$^{-/-}$ EBs transfected with control vector (n=3). **F**, Anti-Flag immunoprecipitates of nuclear extracts from Jmjd3-Flag overexpressing HEK293 cells were analyzed by anti-β-catenin immunoblotting. Five percent of total lysates were loaded as input. **G**, Luciferase reporter assay in WT and *Jmjd3*$^{-/-}$ ESCs transfected with lymphoid enhancer binding factor 1 (LEF1) luciferase reporter construct, LEF1, β-catenin harboring a nuclear localization signal, and a rous sarcoma virus (RSV)-β-galactosidase expression construct. The luciferase levels were normalized for the β-galactosidase activity of the cotransfected RSV-β-galactosidase reporter and represented as fold change relative to the luciferase level from WT ESCs transfected with the same constructs (n=3). **H**, Summary of results. **P*<0.05, ***P*<0.01, and ****P*<0.001. NLS indicates nuclear localization signal.

(Online Figure VII). This is consistent with the requirement of Jmjd3 for blastocyst development,[27] but is in contrast to other studies which showed that Jmjd3-deficient mice are perinatal lethal.[12,28] The discrepancy between the phenotypes of *Jmjd3*$^{-/-}$ mice is unclear but might be related to the different strategies that were used to generate the *Jmjd3*$^{-/-}$ mice. For example, the study of Burgold et al[28] resulted only in a half maximal reduction of *Jmjd3* expression, whereas *Jmjd3* expression is fully absent in our mice. The early embryonic lethality of our mice, however, precluded the analysis of the effect of Jmjd3 on mesoderm development in vivo. Further studies involving conditional deletion of Jmjd3 using Sox2- and Brachyury-Cre lines are required.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Cell fate decisions require well-controlled changes in gene expression that are tightly controlled by epigenetic modulators.
- The post-transcriptional modifications of histone proteins epigenetically regulate gene expression.
- Trimethylation of lysine 27 at histone K3 (H3K27me3) silences gene expression and can be reversed by the demethylase Jmjd3.

What New Information Does This Article Contribute?

- The histone demethylase Jmjd3 is required for mesoderm differentiation and cardiovascular lineage commitment of mouse embryonic stem cells.
- This effect is partially mediated by a silencing of the mesodermal regulator Brachyury.
- Ablation of *Jmjd3* further reduces β-catenin recruitment to the Brachyury promoter, which interferes with Wnt signaling that is required for proper mesoderm differentiation.

The differentiation of stem cells to specific lineages requires a well-defined modulation of gene expression programs, which is often controlled by epigenetic mechanisms. Although several epigenetically active enzymes and complexes have been described, the function of the histone demethylase Jmjd3 for cardiovascular lineage commitment was unknown. Using mouse embryonic stem cells as a model, we now show that the demethylase Jmjd3 is required for mesoderm differentiation and for the differentiation of embryonic stem cells to the vascular and cardiac lineage. We further identified the mechanism and showed that ablation of *Jmjd3* resulted in a silencing of the Brachyury promoter that is associated with an increase in H3K27me3 marks. In addition, Jmjd3 was shown to facilitate the recruitment of β-catenin to the Brachyury promoter, which contributes to the Wnt-dependent activation of mesoderm differentiation. Together these data describe a novel epigenetic mechanism that controls cell fate decision.