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The Histone Demethylases Jhdm1a/1b Enhance Somatic Cell Reprogramming in a Vitamin-C-Dependent Manner

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SUMMARY

Reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) resets the epigenome to an embryonic-like state. Vitamin C enhances the reprogramming process, but the underlying mechanisms are unclear. Here we show that the histone demethylases *Jhdm1a/1b* are key effectors of somatic cell reprogramming downstream of vitamin C. We first observed that vitamin C induces H3K36me2/3 demethylation in mouse embryonic fibroblasts in culture and during reprogramming. We then identified *Jhdm1a/1b*, two known vitamin-C-dependent H3K36 demethylases, as potent regulators of reprogramming through gain- and loss-of-function approaches. Furthermore, we found that *Jhdm1b* accelerates cell cycle progression and suppresses cell senescence during reprogramming by repressing the *Ink4/Arf* locus. *Jhdm1b* also cooperates with *Oct4* to activate the microRNA cluster 302/367, an integral component of the pluripotency machinery. Our results therefore reveal a role for H3K36me2/3 in cell fate determination and establish a link between histone demethylases and vitamin-C-induced reprogramming.

INTRODUCTION

Somatic cell fate can be reset into a pluripotent-like state by introducing defined factors known to regulate pluripotency, including *Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog*, and *Lin28* (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). This process is normally referred to as nuclear reprogramming or more simply reprogramming. The resultant cells are termed iPSCs and are similar to embryonic stem cells (ESCs) in many aspects, including gene expression profile, epigenetic marks, and development potential (Kang et al., 2009; Maherali et al., 2007; Zhao et al., 2009). As such, iPSCs derived from human subjects have multiple potential applications ranging from disease modeling for both drug screening and pathway mapping to cell replacement therapies (Zhang et al., 2011; Zhou et al., 2011). Besides, the reprogramming process may serve as a paradigm for understanding cell fate determination at the molecular level.

Reprogramming has been so far approached in multiple ways mechanistically (Plath and Lowry, 2011). For example, Hochedlinger and colleagues showed that downregulation or upregulation of markers such as Thy1 or SSEA-1 occurs at an early stage, but activation of pluripotent genes and X chromosome inactivation are late events (Stadtfeld et al., 2008). By analyzing the genome-wide DNA binding patterns of the four Yamanaka factors (Takahashi and Yamanaka, 2006), Plath and colleagues also delineated the specific contribution of each factor and uncovered a temporal and separable mode of action (Sridharan et al., 2009). On the other hand, we and others have shown that a mesenchymal-to-epithelial transition (MET) initiates and is necessary for the reprogramming of mouse fibroblasts (Li et al., 2010; Lin et al., 2009; Samavarchi-Tehrani et al., 2010). We also showed that microRNA cluster 302/367 promotes reprogramming by accelerating MET, among other events (Liao et al., 2011; Subramanyam et al., 2011). All together, these studies have provided a conceptual framework for understanding reprogramming at the molecular and cellular level.

The epigenetic state of a cell is determined by the unique pattern of DNA methylation and histone modifications, and is responsible for cell- and tissue-specific gene expression patterns. Reprogramming must thus involve mechanisms responsible for changing the epigenetic status of both DNA and histones genome-wide. Interestingly, despite the fact that the acquisition of the pluripotent state during reprogramming entails both DNA methylations at the promoters of somatic genes (Lister et al., 2009) and the DNA demethylations at the promoters of pluripotent genes, the two de novo DNA methyltransferases *Dnmt3a* and *b* are dispensable (Pawlak and Jaenisch, 2011), suggesting that additional epigenetic enzymes/modifiers play a relevant role. In this regard, the histone deacetylase inhibitors valproic acid, suberoylanilide hydroxamic acid, and sodium butyrate are known to enhance reprogramming (Huangfu et al., 2008; Liang et al., 2010; Mali et al., 2010), suggesting that histone deacetylases may represent a roadblock for iPSC generation. The chromatin remodeling proteins Bgr1 and Baf155, along with Wdr5, an effector of H3K4 methylation, also have potentiating effect (Ang et al., 2011; Singhal et al., 2010). Moreover, Meissner and colleagues analyzed the global euchromatin dynamics in the initial phase of reprogramming and observed

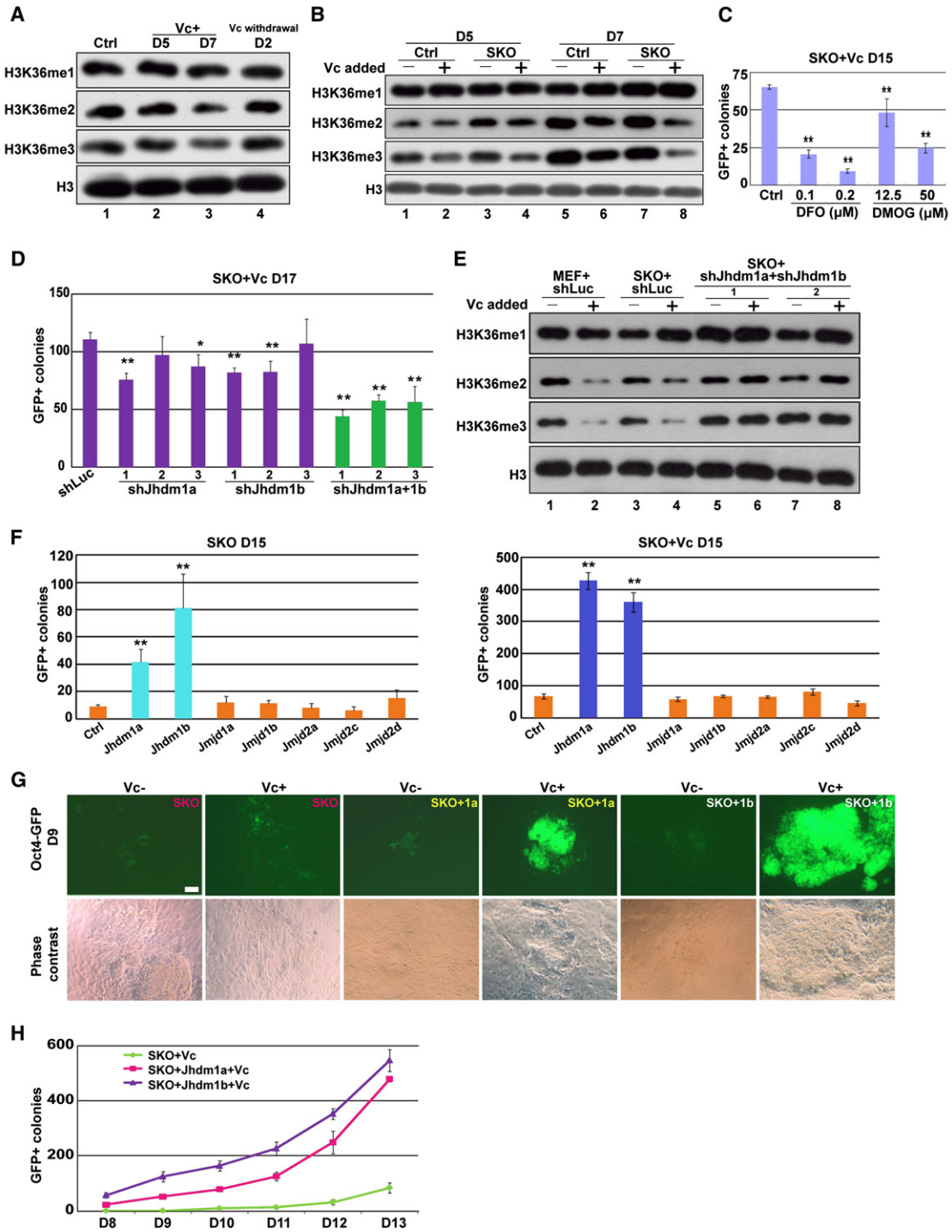

Figure 1. Vitamin C Promotes the Demethylation of H3K36me2/me3 and Accelerates Reprogramming Synergistically with *Jhdm1a/1b*

(A) Vitamin C (Vc) reduces H3K36me2/3 in mouse fibroblasts in a reversible manner. Ctrl and D indicate control and day, respectively. A representative western blot is shown.

(B) Vitamin C reduces H3K36me2/3 modification during reprogramming with three factors. Control fibroblasts were infected with *GFP* retroviruses (this applies hereafter); the total number of viruses was kept constant between experiments. A representative western blot is shown. Total histone 3 was used as loading control.

(C) DFO and DMOG inhibit SKO reprogramming. The two compounds were added to the media along with vitamin C at indicated concentrations through the whole experiment. Mean values ± standard deviation (SD) of three independent experiments are shown; this also applies hereafter for any experiment analyzing reprogramming efficiency. **$p < 0.01$ with one-way AVONA analysis.

(D) Reprogramming efficiency with SKO + vitamin C is impaired by shRNA (sh) for *Jhdm1a/1b*. shLuc indicates shRNA against firefly luciferase gene; 1a indicates *Jhdm1a*, 1b indicates *Jhdm1b*. *$p < 0.05$.

an orchestrated epigenetic response to ectopic factors at the histone modification level (Koche et al., 2011). Taken together, these findings show that chromatin remodeling during reprogramming is organized rather than random, and is also susceptible to acceleration.

We previously reported that vitamin C promotes the generation of mouse and human iPSCs (Esteban et al., 2010). Yet, other antioxidants, including N-acetylcysteine, vitamin E, and lipoic acid, have little effect even though they also reduce the level of free radicals, suggesting that vitamin C may have additional functions other than that of suppressing free radicals. In fact, vitamin C critically regulates cellular functions such as collagen synthesis (Van Robertson and Schwartz, 1953) and hypoxia-inducible factor stability (Knowles et al., 2003), and also has been ascribed additional roles in areas ranging from cancer to the common cold (Traber and Stevens, 2011; Wilson and Loh, 1973). In this report, we demonstrate that vitamin C enhances iPSC generation at least in part by facilitating the function of histone demethylases *Jhdm1a/1b*.

RESULTS

Vitamin C Promotes the Demethylation of H3K36me2/me3 and Accelerates Reprogramming Synergistically with *Jhdm1a/1b*

Vitamin C is a known cofactor for multiple histone demethylases (Horton et al., 2010), and this led us to hypothesize that mouse fibroblasts reprogrammed in the presence of vitamin C undergo changes in the histone methylation status that accelerate the process. To test this idea, we extracted total histones from fibroblasts treated with vitamin C at different time points and analyzed the methylation status by western blotting using antibodies against monomethylated, dimethylated, and trimethylated histone H3 at K4, K9, K27, and K36. Vitamin C consistently reduced the levels of H3K36me2/3 at days 5 and 7 posttreatment (Figure 1A and Figure S1A available online), whereas other tested histone marks did not change noticeably (Figure S1B). Importantly, when vitamin C was removed from the culture medium, the level of H3K36me2/3 returned to that of the steady state in 2 days (Figure 1A and Figure S1A), indicating that vitamin C modulates H3K36 methylation in a reversible way. Vitamin C also decreased H3K36me2/3 levels, but not other tested histone marks, during reprogramming mediated by *Sox2/Klf4/Oct4* (SKO) (Figure 1B and Figures S1C and S1D). We then treated fibroblasts transduced with SKO with the iron chelator desferrioxamine (DFO) or the α-ketoglutarate analog dimethyloxalylglycine (DMOG) to test whether the changes in H3K36me2/3 are driven by vitamin C/iron/α-ketoglutarate-dependent dioxygenases (e.g., the Jumonji family of histone demethylases) (Horton et al., 2010). DFO and DMOG inhibited SKO reprogramming in a dose-dependent manner, reducing cell proliferation moderately (Figure 1C and Figure S1E). Similar results were obtained with SKOM (the same factors plus c-Myc) (Figure S1F). These results suggested that vitamin C influences reprogramming by removing H3K36me2/3 through vitamin-C-dependent histone demethylases.

Jhdm1a/1b (sharing 56% homology) belong to the Jumonji family of proteins and have been shown to demethylate H3K36me2/3 (He et al., 2008). We analyzed the mRNA level of *Jhdm1a/1b* in fibroblasts transduced with SKO or SKOM and found a moderate increase over the time course of reprogramming with SKOM (Figure S2A left panel). Likewise, the expression of these two enzymes was 5- to 8-fold greater in ESCs than in fibroblasts (Figure S2A left panel). Other tested demethylases showed variable increase during reprogramming with SKO/SKOM and were also expressed at greater levels in ESCs than in fibroblasts (Figure S2A right panel). To analyze the role of *Jhdm1a/1b* in reprogramming, we knocked them down using shRNA retroviral vectors. The effect of reducing *Jhdm1a* or *1b* expression independently was moderate, but the combined knockdown impaired reprogramming efficiency significantly (Figure 1D and Figure S2B). Importantly, there was a concomitant increase in the levels of H3K36me2/3 (Figure 1E and Figure S2C), in agreement with vitamin C reducing this histone mark through *Jhdm1a/1b*. Knockdown of *Jhdm1a/1b* also inhibited reprogramming in the absence of vitamin C (Figure S2D). This further shows that these two enzymes are critical for reprogramming but also indicates that they can work, albeit likely less efficiently, without vitamin C.

We then performed gain-of-function studies by overexpressing *Jhdm1a/1b* and a few additional histone demethylases together with SKO. *Jhdm1a/1b*, but not the other enzymes, enhanced iPSC generation with SKO in the presence (more significantly) or absence of vitamin C (Figure 1F), and less significantly with SKOM (Figure S2E). *Jhdm1a/1b* also accelerated the reprogramming kinetics in the presence of vitamin C (Figures 1G and 1H). As expected, DFO and DMOG also counteracted the enhancing effect of *Jhdm1a/1b* on reprogramming (Figure S2F). Our results thus suggest that *Jhdm1a/1b* play a relevant role in vitamin-C-enhanced reprogramming.

Jhdm1b Enables Efficient Generation of iPSCs by *Oct4* Alone

We and others have shown that *Oct4* is sufficient to reprogram human and mouse fibroblasts into iPSCs, albeit at extremely low efficiencies (Chen et al., 2011; Li et al., 2011; Zhu et al., 2010). We tested the possibility that removal of H3K36me2/3 marks by *Jhdm1a/1b* would enhance reprogramming efficiency by *Oct4* alone. We transduced mouse fibroblasts with KO, SO, or O, plus either *Jhdm1a* or *1b*, and then treated the reprogrammed

(E) shRNA for *Jhdm1a/1b* reverse the reduction in H3K36me2/3 triggered by SKO and vitamin C. MEF indicates mouse embryonic fibroblasts used as a control. A representative western blot is shown.
(F) Overexpression of *Jhdm1a/1b* enhance reprogramming efficiency with SKO in the presence or absence of vitamin C.
(G) Phase contrast and immunofluorescence microscopy of fibroblasts transduced and treated as indicated. 1a indicates *Jhdm1a*. Scale bar corresponds to 45 μm.
(H) *Jhdm1a/1b* greatly accelerate the kinetics of GFP+ colony appearance in fibroblasts transduced with SKO. A representative time course experiment is shown. See also Figures S1 and S2.

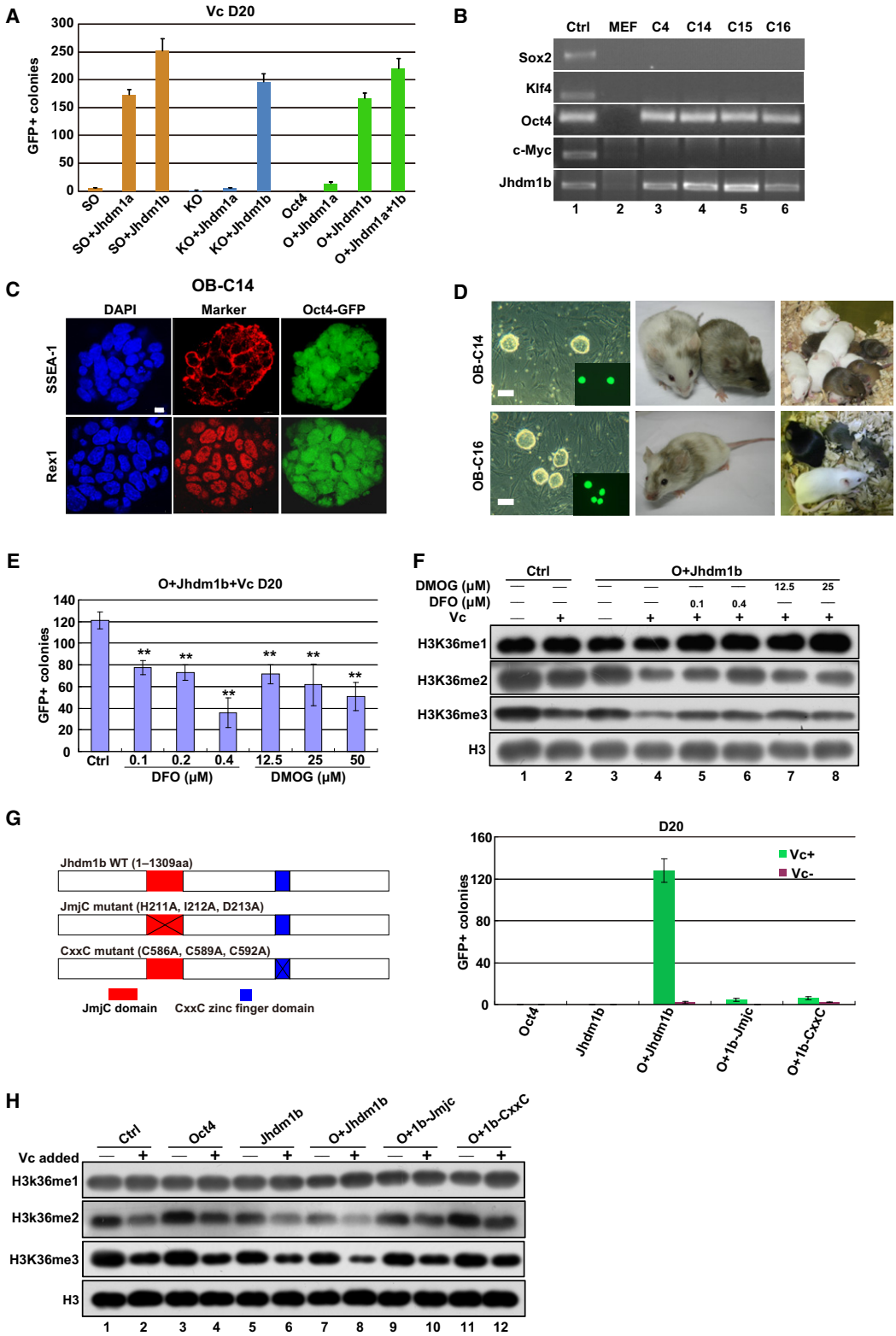

Figure 2. *Jhdm1b* Enables Efficient Generation of iPSCs by *Oct4* Alone and This Requires Histone Demethylase Activity

(A) Effect of *Jhdm1a*/*1b* on the reprogramming of mouse fibroblasts with fewer factors.

(B) Semiquantitative RT-PCR shows integration of the transgenes into the genome of selected OB clones.

(C) Confocal immunofluorescence microscopy for the indicated markers using a selected OB clone. Nuclei are stained in blue with DAPI. Scale bar corresponds to 10 μm.

cells with vitamin C. *Jhdm1a/1b* enabled efficient reprogramming mediated by SO, but only *Jhdm1b* boosted KO-mediated reprogramming significantly (Figure 2A). These data indicate that although *Jhdm1a/1b* may share some functions, their mode of action is not identical. In addition, *Jhdm1b* promoted *Oct4* reprogramming to a level similar to that of KO or SO plus *Jhdm1b*, reaching an efficiency of 0.85% (~170 clones derived from 20,000 cells) at day 20 (Figure 2A), while *Jhdm1a* could only produce a few clones. This efficiency is greater than OK-mediated reprogramming under optimized tissue culture conditions (0.02%) (Chen et al., 2010). We referred to clones derived from *Oct4* and *Jhdm1b* as OB iPSCs. These OB clones had integrated *Oct4* and *Jhdm1b* into the genome, and we verified that *Sox2*, *Klf4*, and *c-Myc* transgenes were absent (Figure 2B). They also displayed normal karyotype, silencing of *Oct4* and *Jhdm1b* transgenes, activation of endogenous pluripotent markers, and demethylation of *Oct4* and *Nanog* proximal promoters (Figures S3A–S3E). Immunofluorescence for REX1 and SSEA-1 was likewise comparable to that of mouse ESCs (Figure 2C), and injection into eight-cell stage wild-type embryos produced live-born chimeras with germline transmission (Figure 2D). Therefore, *Jhdm1a/1b* differ in their ability to enhance the reprogramming of mouse fibroblasts by two factors or *Oct4* alone.

Jhdm1b Enhances *Oct4* Reprogramming through Its Histone Demethylase Activity

Next, we aimed to understand how *Jhdm1b* enhances *Oct4* reprogramming, as this should explain to a large extent how it works with two, three, or four factors. Our results above using DFO and DMOG suggested that *Jhdm1a/1b* enhance iPSC generation through their demethylase activity. These two inhibitors impaired as well the reprogramming of fibroblasts transduced with *Oct4*/*Jhdm1b* and treated with vitamin C (Figure 2E). Likewise, we observed a significant reduction of H3K36me2/3 in *Oct4*/*Jhdm1b*-transduced fibroblasts treated with vitamin C as compared with the control-treated cells (Figure 2F lane 2 versus 4 and Figure S4A), and this was reversed by DFO and DMOG (Figure 2F lanes 2 and 4 versus 5–8, and Figure S4A). Moreover, we constructed two *Jhdm1b* mutants lacking either the DNA binding domain (named CxxC mutant) or the catalytic domain (Jmjc mutant) respectively (Figure 2G left panel). The zinc finger CxxC domains of *Jhdm1a/1b* have been shown recently to preferentially recognize nonmethylated DNA and then remove H3K36me2/3 marks (Blackledge et al., 2010). We showed that both mutants failed to enhance iPSC generation by *Oct4* in the presence or absence of vitamin C (Figure 2G right panel). As expected, we also showed that both mutants also lacked the ability to demethylate H3K36me2/3 in the context of reprogramming, unlike the wild-type (Figure 2H lane 6 and 8 versus 9–12, Figure S4B). Hence, we conclude that *Jhdm1b* promotes *Oct4* reprogramming through its histone demethylase activity.

Jhdm1b Promotes Cell Cycle Progression and Overcomes Cell Senescence during Reprogramming

The significant changes in H3K36me2/3 methylation driven by *Jhdm1b* and vitamin C suggest that such genome-wide epigenetic rearrangements may favor the reprogramming process. Previous reports have described a role for *Jhdm1b* in regulating fibroblast proliferation and senescence through H3K36me2/3 demethylation (He et al., 2008; Tzatsos et al., 2009). Consistent with this report, we showed that *Jhdm1b* transduction increased fibroblast proliferation (Figure 3A). Treatment with vitamin C enhanced proliferation as well, as reported previously before (Esteban et al., 2010), and the combination of *Jhdm1b* and vitamin C was highly synergistic (Figure 3A). Conversely, the knockdown of both *Jhdm1a/1b* significantly inhibited cell growth even in the presence of vitamin C (Figure 3B, left and right panels). We also performed DNA microarray analysis of *Jhdm1b*-transduced fibroblasts treated with vitamin C and observed that cell cycle regulators were among the most upregulated genes (Figure 3C). Next, we analyzed the effect of *Jhdm1b*/vitamin C on cell proliferation in the context of *Oct4* reprogramming. To our surprise, the transduction of *Oct4* alone in fact inhibited the proliferation of fibroblasts (Figures 3D and 3E), increased the number of G1 cells, and decreased the number of cells in S and G2/M (Figure 3F), compared with the control. The proliferation arrest was overcome by *Jhdm1b*/vitamin C (Figures 3D and 3E), which also reduced the number of cells in G1 phase and increased the number of S and G2/M cells in the presence or absence of *Oct4* (Figure 3F). We then analyzed the expression of cell cycle regulators through qPCR and confirmed that *Jhdm1b*/vitamin C significantly upregulated key cell cycle regulators in *Oct4*-transduced fibroblasts (Figure 3G).

Reprogramming is proliferation dependent, which allows the progressive acquisition of a pluripotent status (Hanna et al., 2009), and the *Ink4/Arf* locus acts as a roadblock in this process by inducing cell senescence (Li et al., 2009). It is known that *Jhdm1b* enhances fibroblast proliferation through repression of the *Ink4/Arf* locus by removing H3K36me2/3 marks (He et al., 2008; Tzatsos et al., 2009). We tested the idea that *Jhdm1b* and vitamin C promote *Oct4* reprogramming by overcoming *Ink4/Arf*-triggered cell senescence. We performed reprogramming of fibroblasts at early or late passage in the presence of *Jhdm1b*, vitamin C, or both. Control cells underwent senescence rapidly and became resistant to reprogramming at passage 6 (P6) (Figures 4A and 4B). Vitamin C or *Jhdm1b* alone partially rescued the reprogramming of P6 fibroblasts, and their

(D) Generation of chimeric mice with germline transmission after blastocyst injection of iPSCs from selected OB clones. Phase contrast and immunofluorescence photographs for the same clones are shown on the left; scale bar corresponds to 45 μm.
(E) DFO and DMOG inhibit reprogramming with *Oct4*/*Jhdm1b*/vitamin C.
(F) shRNA for *Jhdm1a/1b* reverses the reduction in H3K36me2/3 triggered by *Oct4*/*Jhdm1b*/vitamin C.
(G) Left: scheme depicting the generation of *Jhdm1b* mutants lacking histone demethylase or DNA binding activity. Right: both mutants lack the ability to enhance *Oct4* reprogramming with vitamin C, unlike wild-type control. Jmjc and CxxC indicate Jhdm1b mutants lacking the histone demethylase and DNA binding motif, respectively.
(H) Jmjc and CxxC mutants also lack the ability to reduce H3K36me2/3 during reprogramming with *Oct4* and vitamin C, unlike wild-type control.
See also Figures S3 and S4 and Table S1.

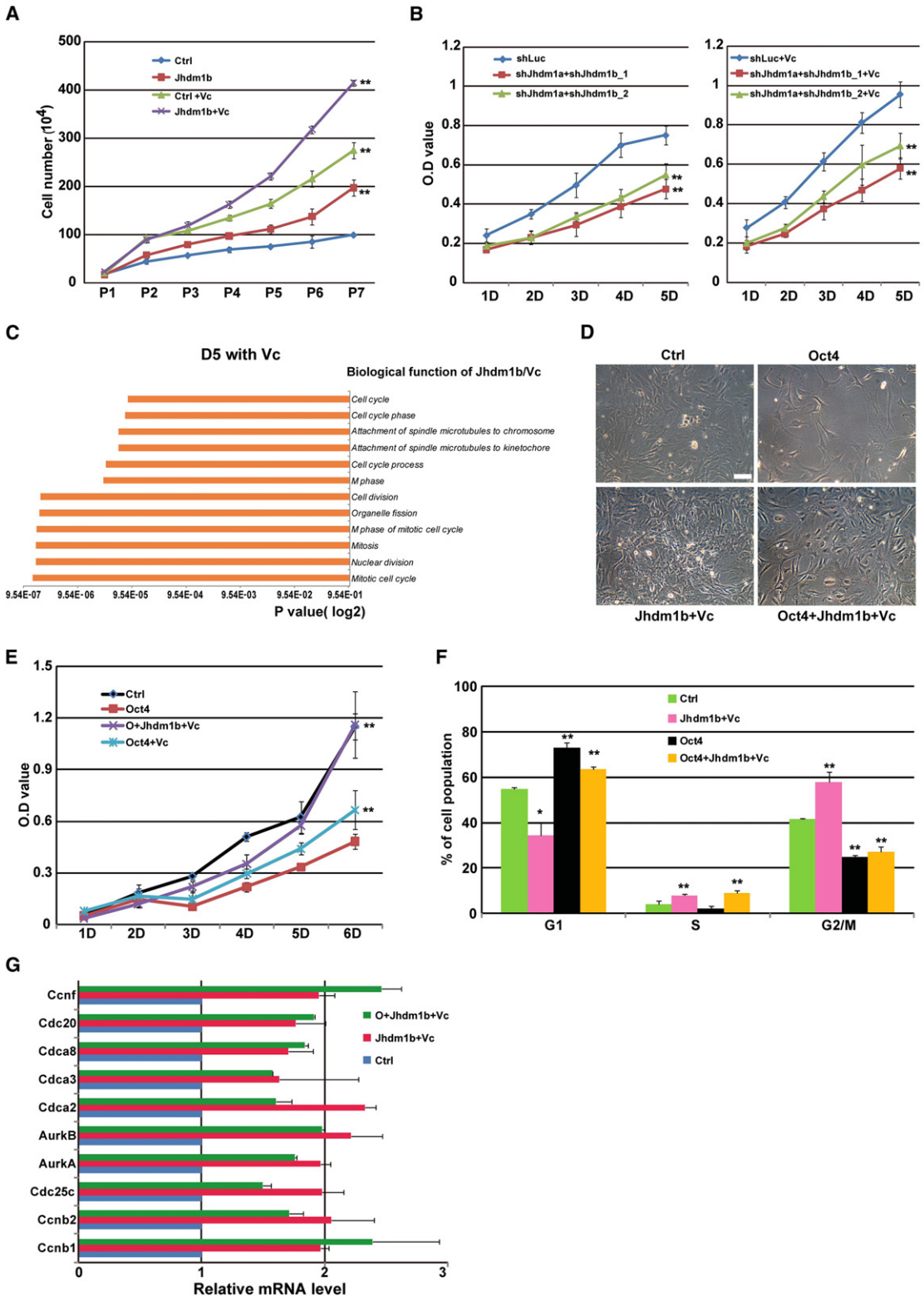

Figure 3. *Jhdm1b* Promotes Cell Cycle Progression during Reprogramming

(A) Fifty thousand fibroblasts were plated on 6-well plates and transduced/treated as indicated, and the cell number was measured before passaging. A representative experiment performed in triplicate (each well was counted two times) is shown.

(B) Nine hundred fibroblasts transduced as indicated in a 96-well plate; cell proliferation was measured at different time points using MTT. A representative experiment out six repeats performed is shown.

combination effectively restored it (Figures 4A and 4B). We also established fibroblasts stably expressing *Jhdm1b* that could be reprogrammed at P6 either with vitamin C (more efficiently) or without it (Figures 4A and 4B). ChIP analysis confirmed that *Jhdm1b* binds to different locations in the *Ink4/Arf* locus in cells transduced with *Oct4/Jhdm1b* (Figure 4C) (He et al., 2008; Tzatsos et al., 2009). Moreover, *Jhdm1b*/vitamin C reduced the level of H3K36me2 at positions 3 (more significantly), 6, and 10 of the *Ink4/Arf* locus, while the repressive mark H3K27me3 was increased (Figure 4D). Consistently, mRNA for $p16^{Ink4a}$ increased in P6 fibroblasts compared with P2, but was potently reduced in the presence of *Jhdm1b* (Figure 4E). As for $p15^{Ink4b}$, the protein level was upregulated in late passage (P4 versus P2) and was further increased by *Oct4* alone (Figure 4F), which may help explain the above-described inhibitory effect of *Oct4* on fibroblast proliferation (Figures 3D–3F). Next, we induced cell senescence by overexpressing *p15* and observed a strong inhibitory effect on reprogramming that could be partially reversed by *Jhdm1b*/vitamin C (Figure 4G). These data suggest that *Jhdm1b* promotes *Oct4* reprogramming by repressing the *Ink4/Arf* locus, which may allow continued proliferation by overcoming cell senescence.

Jhdm1b Interacts with Oct4 to Activate the Expression of microRNA Cluster 302/367 during Reprogramming

ESC-specific microRNAs, including the microRNA cluster 302/367, play an essential role in maintaining the ESC cell cycle (Wang et al., 2008). Recent reports have shown that components of this microRNA cluster promote reprogramming mediated by SKO (Liao et al., 2011; Subramanyam et al., 2011) and can produce mouse and human iPSCs without additional factors (Anokye-Danso et al., 2011; Miyoshi et al., 2011). Of note, microRNA cluster 302/367 is a downstream target of *Oct4* in mouse ESCs (Barroso-delJesus et al., 2008). Therefore, we investigated whether *Jhdm1b* and *Oct4* cooperate to activate the expression of this cluster, as this may further explain the effects of *Jhdm1b* on cell proliferation during *Oct4* reprogramming. Interestingly, *Oct4* or *Jhdm1b* alone failed to induce this microRNA cluster significantly in transduced fibroblasts, but the combination of *Oct4/Jhdm1b* plus vitamin C activated it efficiently (Figure 5A and Figure S5A). We also observed that transfection of microRNA-302/367 antagonists (antagomirs) impaired the reprogramming of fibroblasts transduced with SKO and *Jhdm1b* (Figure 5B), indicating that activation of these microRNAs is required for *Jhdm1b*-enhanced reprogramming.

We investigated the mechanism through which *Oct4* and *Jhdm1b* regulate the expression of microRNA cluster 302/367 by cloning the corresponding promoter upstream of the firefly luciferase reporter gene (Figure 5C). We observed that these two factors cooperate to activate this promoter (Figure 5C), suggesting a physical interaction between them. We also performed ChIP-qPCR on cells infected with *Oct4* alone or *Oct4* together with *Jhdm1b* (wild-type or mutant) in the presence of vitamin C. Oct4 alone failed to bind to the endogenous microRNA cluster 302/367 promoter at its known binding site, but coexpression with wild-type *Jhdm1b* (not the mutated version) induced significant binding (Figure 5D). This suggests that putative histone modifications catalyzed by Jhdm1b in the nearby chromatin are required for Oct4 binding and target activation. To search for putative binding sites of Jhdm1b on the microRNA cluster 302/367 promoter, we designed primer sets that span the entire promoter region. We performed ChIP-qPCR analysis to show that Jhdm1b binds to a region near the Oct4 site upon coexpression with *Oct4* (Figure 5E and Figure S5B). Moreover, Oct4 and Jhdm1b could be coimmunoprecipitated upon cotransfection in HEK293T cells (Figure S5C). We observed a reduction of H3K36me2 (more remarkably) and H3K36me3 in the regions surrounding the Oct4 and Jhdm1b binding sites (Figure 5F). These data suggest that Jhdm1b and Oct4 interact with each other to activate microRNA cluster 302/367, which in turn facilitates reprogramming.

DISCUSSION

Vitamin C is a natural compound essential to human health (Traber and Stevens, 2011). It acts at least in part as a cofactor for enzymatic reactions involving iron and α-ketoglutarate-dependent dioxygenases. As such, it is necessary for the function of collagen prolyl hydroxylases, and its deficiency causes lack of extracellular collagen assembly, leading to scurvy (Peterkofsky, 1991). Other susceptible enzymes include Hypoxia-Inducible Factor prolyl hydroxylases (Knowles et al., 2003) and many histone demethylases (Horton et al., 2010). We have reported recently that vitamin C accelerates and enhances somatic cell reprogramming with exogenous factors (Esteban et al., 2010), a function not ascribable to its antioxidant activity alone. We then hypothesized in this study that the effect of vitamin C may rely at least in part on its ability to enhance histone demethylase function, but proof of evidence was missing (Esteban et al., 2010). Interestingly, it has also been reported that vitamin C promotes widespread DNA CpG demethylation in human ESCs through an unknown mechanism (Chung et al., 2010), further suggesting a link between vitamin C and epigenetic remodeling. In this report, we presented evidence that vitamin C promotes the histone demethylase activities of *Jhdm1a/1b* during enhanced reprogramming, and as such, their knockdown impairs iPSC generation significantly. At this point, we cannot exclude the possibility that vitamin C and/or *Jhdm1a/1b* promote reprogramming through alternative mechanisms. The role of histone demethylases, including members of the Jumonji family of proteins, in cell fate decisions is well established in multiple contexts. For example, *LSD1* (which does not

(C) Summary of enriched Gene Ontology (GO) terms more potently activated by *Jhdm1b*/vitamin C; the DNA arrays were performed with cells at passage 1.
(D) Phase contrast photographs of cells transduced/treated as indicated. Scale bar is 95 μm.
(E) *Jhdm1b* with vitamin C rescues the cell cycle arrest triggered by *Oct4* transduction into fibroblasts. A representative experiment out six repeats performed is shown.
(F) Cell cycle analysis with propidium iodide and flow cytometry confirms the results in (E).
(G) qPCR analysis of the indicated genes in cells transduced as indicated. The control are cells transduced with *GFP* retroviruses.
See also Table S1.

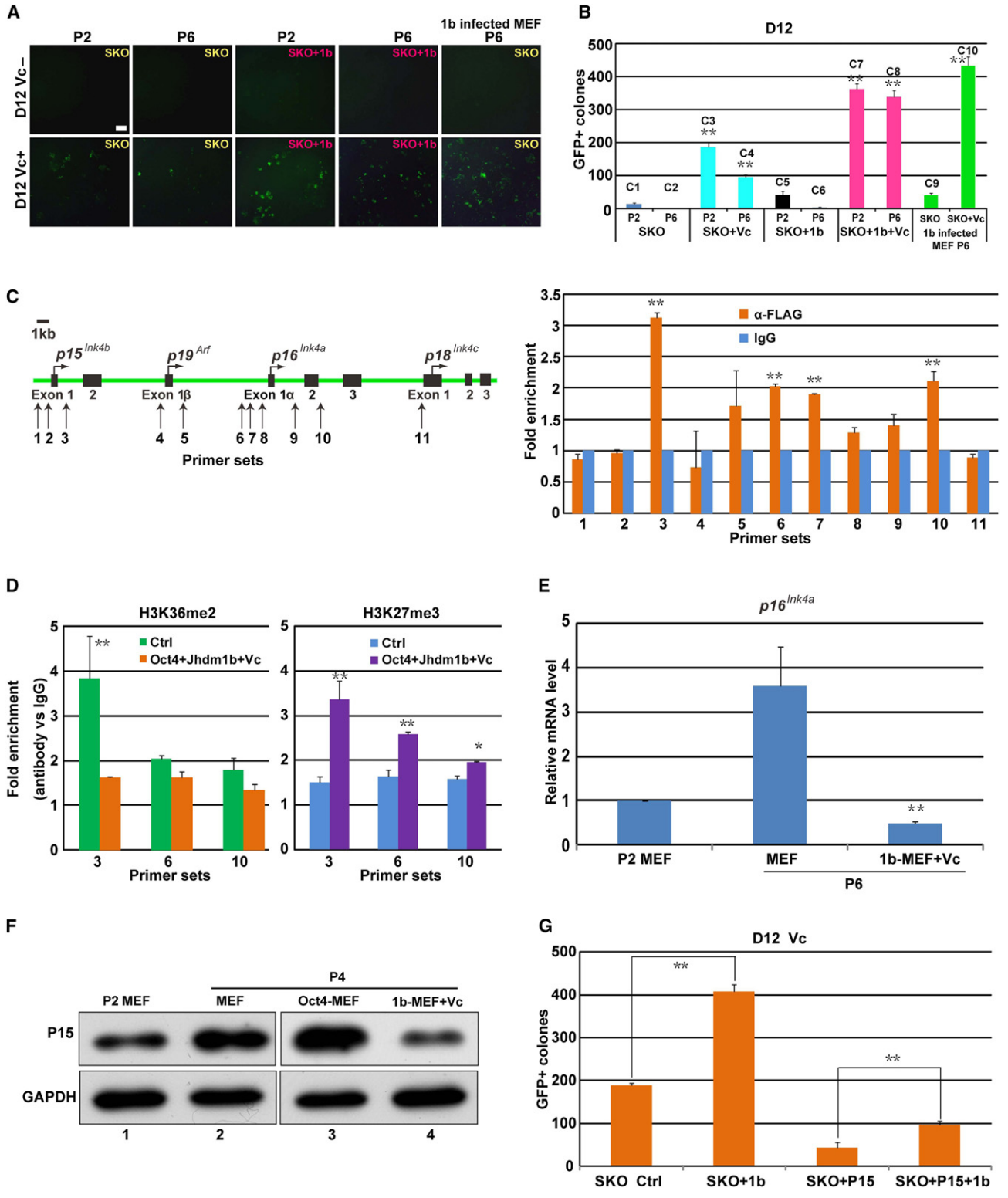

Figure 4. *Jhdm1b* Overcomes Cell Senescence during Reprogramming by Repressing the *Ink4/Arf* Locus

(A and B) *Jhdm1b*/vitamin C overcome the cell senescence roadblock during reprogramming of late passage fibroblasts. Appearance of GFP+ colonies in fibroblasts transduced/treated as indicated are shown in (A), and the reprogramming efficiency of three independent experiments is measured in (B). Scale bar indicates 95 μm.

(C) Left: schematic representation of the *Ink4/Arf* locus. Right: analysis of *Jhdm1b* binding in this locus using ChIP-qPCR. Mean values of three independent experiments ± SD are included.

need vitamin C for function) regulates self-renewal and differentiation in human ESCs (Adamo et al., 2011). Likewise, *Jmjd1a* is important for *Oct4* reactivation in cell-fusion-mediated reprogramming (Ma et al., 2008). However, a role for vitamin C/iron/α-ketoglutarate histone demethylases in somatic cell reprogramming had not yet been reported. Our results also show that the overexpression of *Jhdm1a/1b* potently enhances reprogramming with three (SKO) or fewer factors in the presence of vitamin C, and *Jhdm1b* can efficiently produce iPSCs with *Oct4* alone. This is relevant from a practical point of view because using only one exogenous transcription factor for inducing reprogramming may allow improved strategies for understanding the process mechanistically. Moreover, we have demonstrated that vitamin C induces a global change in the levels of H3K36me2/3 during reprogramming, suggesting that the elimination of these histone marks in at least some loci is necessary for iPSC generation. In this regard, we demonstrate that removal of H3K36me2/3 in the *Ink4/Arf* locus and the promoter of microRNA cluster 302/367 are two relevant mechanisms by which *Jhdm1b* enhances reprogramming (Figure 6). Activation of the former locus happens naturally during somatic cell senescence in vitro and is a well-known roadblock of reprogramming (Li et al., 2009). Our results describing repression of *Ink4/Arf* by *Jhdm1b* and prevention of fibroblast senescence are consistent with published results outside the context of reprogramming (He et al., 2008; Tzatsos et al., 2009). This observation also supports our own data showing that vitamin C enhances iPSC generation at least in part by delaying cell senescence (Esteban et al., 2010). As for microRNA cluster 302/367, it is known that proper upregulation of its components is necessary for reprogramming and that they favor the process through multiple mechanisms, including repression of the *Tgfβ* signaling pathway and cell cycle modulation (Liao et al., 2011; Subramanyam et al., 2011). Interestingly, binding and activation of this microRNA cluster by Oct4 needs the removal of H3K36me2/3 marks by Jhdm1b, indicating that changes in this histone modification mark can be associated with both gene activation and gene repression (*Ink4/Arf*). It is tempting to speculate that removal of H3K36me2/3 in other loci influences as well reprogramming by unrelated mechanisms, but this needs to be explored further experimentally. It is also interesting to note that the functions of *Jhdm1a/1b* are similar but not identical; for example, only *Jhdm1b* induces *Oct4* reprogramming efficiently. A genome-wide mapping of H3K36 marks along with the binding sites for *Jhdm1a/1b* should be carried out with fine resolution during reprogramming with *Oct4* and vitamin C so that their roles in cell fate determination can be understood in greater detail. Our results also highlight the fact that although the role of H3K36 modifications in activating or repressing gene expression in ESCs is less clear than that for other histone modifications (Bernstein et al., 2006; Mikkelsen et al., 2008), H3K36me2/3 along with their modification enzymes should be investigated further in the context of cell fate determination.

EXPERIMENTAL PROCEDURES

Cell Culture

Mouse embryonic fibroblasts were used throughout the study and were derived from E13.5 embryos carrying the *Oct4-GFP* transgene (Esteban et al., 2010). Fibroblasts and HEK293T cells were maintained in DMEM high glucose (GIBCO) supplemented with 10% FBS (Hyclone). Mouse iPSCs and ESCs were maintained on feeder layers (mouse fibroblasts were treated with mitomycin C) in both mES and KSR media. mES medium consisted of DMEM high glucose supplemented with 15% FBS (GIBCO), glutamine, sodium pyruvate, and LIF (Millipore). KSR medium consisted of DMEM high glucose supplemented with 15% KnockOut Serum Replacement (GIBCO), glutamine, pyruvate, and LIF. Vitamin C (A4034), DFO, and DMOG were purchased from Sigma. For reprogramming experiments vitamin C was used at 50 μg/ml and added from day 2 until the end of each experiment.

Western Blotting

Histones were extracted using a reported method (Shechter et al., 2007). Five micrograms of histone samples were electrophoresized with 15% SDS-PAGE gels. Detection was performed with ECL+ (Amersham). Antibodies against histone modifications were purchased as follows: anti-H3K36me1 (Abcam, ab9048), anti-H3K36me2 (Millipore, 07-274), anti-H3K36me3 (Abcam, ab9050), anti-H3K27me1 (Millipore, 07-448), anti-H3K27me2 (Abcam, ab24684), anti-H3K27me3 (Millipore, 07-449), anti-H3K9me1 (Abcam, ab9045), anti-H3K9me2 (Millipore,17-681), anti-H3K9me3 (Abcam, ab8898), anti-H3K4me1 (Abcam, ab8895), anti-H3K4me2 (Abcam, ab32356), anti-H3K4me3 (Abcam, ab1012), and anti-H3(Abcam, ab1791). P15 and GAPDH antibodies were purchased from Cell Signaling Technology. Anti-Oct4 was purchased from Santa Cruz (SC-8628), and we used Flag antibody (Sigma, F1804) for detection of Jhdm1b (and also for ChIP).

Reprogramming Experiments

pMXs-based retroviral vectors containing the murine cDNAs of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* were purchased from Addgene. Other reprogramming factors including *Jhdm1a* and *Jhdm1b* were cloned to pMXs with PmeI restriction enzyme. These plasmids were transfected into PlatE cells using the calcium phosphate transfection method. OG2 cells within the first 2 passages were split for infection at 3,500 cells per cm^2 for iPSC generation. Infections were carried out as described previously (Esteban et al., 2010). The day that viral supernatants were removed and the medium added was defined as day 0 postinfection. Picked iPSC colonies were cultured as ESCs. Bisulfite genomic sequencing and chimeric mouse generation was performed as described (Esteban et al., 2010).

Plasmids

Human *Jhdm1a* was amplified from H1 cDNA. Murine *Jhdm1b*, *Jmjd1a/1c*, and *Jmjd2a/2c/2d* were amplified from fibroblast cDNA and cloned into PmeI site of pMX vector; all plasmids were sequenced and their expression was confirmed by qPCR. A triplicate flag sequence was tagged at the C-terminal of Jhdm1b for coimmunoprecipitation and ChIP experiments. pRetroSuper vectors containing shRNA sequences for *Jhdm1a* and *Jhdm1b* were designed using the Oligoengine or Sigma website. After infection, cells were cultured in the presence of puromycin for 4 days. shRNA sequences and other primers used in this study are included in Table S1 available online.

(D) ChIP-qPCR analysis of H3K36me2 and H3K27me3 methylation in the *Ink4/Arf* locus of fibroblasts (control) or those transduced with *Oct4/Jhdm1b* and treated with vitamin C. Mean values of three independent experiments ± SD are included.
(E and F) Representative qPCR analysis and western blot for *p16* mRNA and p15 protein, respectively, in fibroblasts transduced/treated as indicated.
(G) *Jhdm1b*/vitamin C partially overcome the reprogramming blockade induced by *p15* coexpression in fibroblasts transduced with SKO.
See also Table S1.

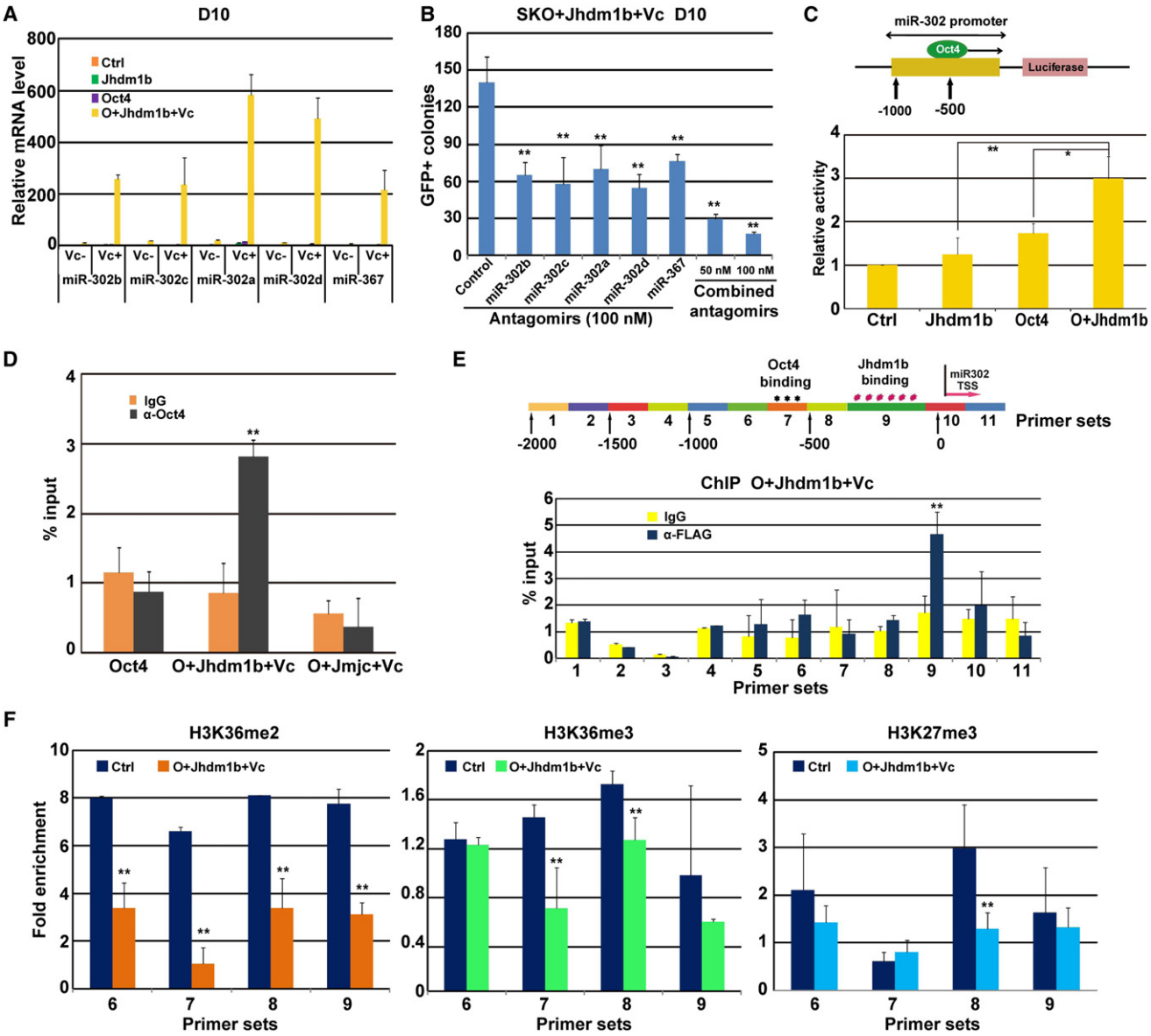

Figure 5. Jhdm1b Interacts with Oct4 to Activate the Expression of microRNA Cluster 302/367 during Reprogramming

(A) Expression measured by qPCR of the indicated microRNA (miR) components in cells transduced/treated as indicated. A representative experiment is included.

(B) Transient transfection of the indicated antagomirs impairs reprogramming efficiency of cells transduced with SKO and *Jhdm1b* plus vitamin C.

(C) Top: scheme of the promoter of microRNA cluster 302/367 cloned upstream of the firefly luciferase gene. Bottom: *Oct4* and *Jhdm1b* cooperate to activate this reporter in HEK293T cells. Mean values ± SD of three independent experiment are shown.

(D) ChIP-qPCR analysis of Oct4 binding to its cognate site on the microRNA cluster 302/367 promoter in cells transduced with *Oct4*/*Jhdm1b* plus vitamin C. Mean values of three independent experiments ± SD are included.

(E) Top: scheme of the promoter for microRNA cluster 302/367 and the different primers used to determine the binding site of *Jhdm1b*. TSS indicates transcription start site. Bottom: ChIP-qPCR analysis using those primers. Mean values of three independent experiments ± SD are included.

(F) ChIP-qPCR analysis of H3K36me2/3 and H3K27me3 in the promoter of microRNA cluster 302/367 in fibroblasts reprogrammed with *Oct4*/*Jhdm1b* plus vitamin C compared with the control. Mean values ± SD of three independent experiments are included.

See also Figure S5 and Table S1.

Immunofluorescence Staining

Immunofluorescence staining was performed as described (Li et al., 2010). Anti-SSEA-1 was purchased from R&D and anti-Rex1 was produced by us. A Leica TCS SP2 Spectral confocal microscope was used for detection.

Proliferation and Cell Cycle Analysis

Cell proliferation assay was performed with MTT method (Beyotime) following standard procedure. Cell cycle analysis was performed when cells reached 80% confluence as detected by using the standard propidium iodide method and flow cytometry analysis.

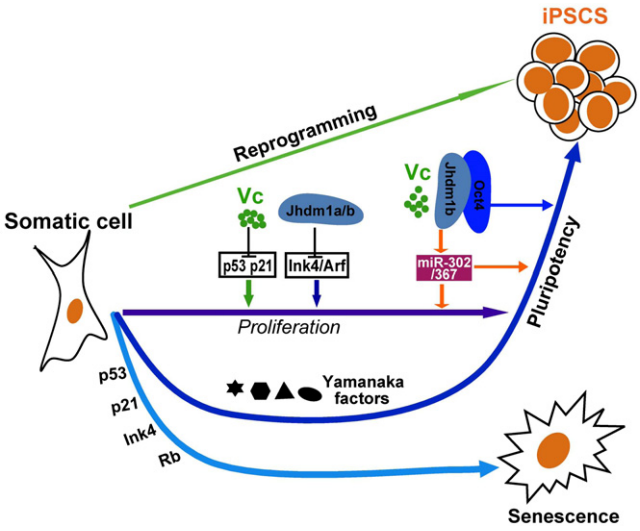

Figure 6. Schematic Representation of How *Jhdm1a/1b* and Vitamin C Enhance Mouse Somatic Cell Reprogramming

Mouse fibroblasts undergo rapid senescence under normal culture conditions and become resistant to reprogramming. The Yamanaka factors can overcome the senescence partially. Vitamin C and *Jhdm1a/1b* can counteract the senescence very effectively by suppressing *p53/p21* (Esteban et al., 2010) and *Ink4/Arf* (this study). The activation of microRNA 302/367 by *Oct4*/*Jhdm1b*/vitamin C facilitates efficient reprogramming.

DNA Microarrays

DNA microarrays were performed using Affymetrix MoGene 1.0 ST chip and were analyzed with Partek software (Partek).

ChIP

This was done following a previously reported protocol (Nelson et al., 2006). ChIP-grade anti-Oct4 and control goat IgG were purchased from Santa Cruz (SC8628X and SC2028), respectively.

Dual Luciferase Reporter Assay

The microRNA cluster 302/367 promoter (1 kb upstream of the transcription start) was amplified from genomic DNA using primers listed in Table S1, and was ligated into the pGL3-basic vector. SV40T-Renilla, the expression vectors for *Oct4* and *Jhdm1b*, and the pGL3-promoter were transfected into HEK293T cells as indicated, and the measurements were performed according to the instructions of the manufacturer (Promega, Dual-Luciferase Reporter Assay System).

microRNA Detection and Antagomir Transfection

Total mRNA was isolated using TRIzol, and 5 μg were used to synthesize cDNA using ReverTra Ace (Toyobo) and oligo-dT (Takara). qPCR was performed using the Premix Ex Taq (Takara) kit and was analyzed with Bio-Rad CFX96 system (Bio-Rad). microRNA assay was detected with stem-loop primers purchased from Ribobio as described (Liao et al., 2011). U6 small nucleolar RNA (Guangzhou RiboBio Co., Ltd) was used for the normalization. All items were measured in triplicate. Antagomirs (Guangzhou RiboBio Co., Ltd) were transfected 1 and 5 days postinfection using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer.

Immunoprecipitation

HEK293T cells were transfected with the indicated plasmids and lysed 36 hr after transfection with TNE buffer (150 mM NaCl, 50 mM Tris-Hcl [pH 7.8], 2 mM EDTA, 1% NP-40, and protease inhibitors) at 4°C for 1 hr. The lysates were centrifuged and only the supernatant was collected. FLAG resin (Sigma, A2220) and anti-Oct4 antibody (Santa Cruz SC-5279) were used for the immunoprecipitation, and protein A/G gel (Santa Cruz, SC-2003) was used to precipitate the antigen/antibody complex. FLAG resin and beads were centrifuged and washed with TNE buffer 10 times at 4°C. For elution we used 50 μl of Flag peptide or SDS buffer, which were incubated with the beads at room temperature for 1 hr; the supernatant was collected by centrifugation.

ACCESSION NUMBERS

The accession number for the DNA microarrays gathered in this study is GSE32994.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Figures S1–S5 and Table S1 and can be found with this article online at doi:10.1016/j.stem.2011.10.005.

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