

Serine 347 Phosphorylation by JNKs Negatively Regulates OCT4 Protein Stability in Mouse Embryonic Stem Cells

Ki Beom Bae,¹ Dong Hoon Yu,¹ Kun Yeong Lee,¹ Ke Yao,¹ Joohyun Ryu,¹ Do Young Lim,¹ Tatyana A. Zykova,¹ Myoung Ok Kim,^{1,2} Ann M. Bode,¹ and Zigang Dong^{1,*}

¹The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912, USA

²The School of Animal BT Science, Kyungpook National University, Sangju, Gyeongsangbuk-do 37224, Republic of Korea

*Correspondence: zgdong@hi.umn.edu

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SUMMARY

The POU transcription factor OCT4 is critical for maintaining the undifferentiated state of embryonic stem cells (ESCs) and generating induced pluripotent stem cells (iPSCs), but its precise mechanisms of action remain poorly understood. Here, we investigated the role of OCT4 phosphorylation in the biological functions of ESCs. We observed that c-Jun N-terminal kinases (JNKs) directly interacted with and phosphorylated OCT4 at serine 347, which inhibited the transcriptional activity of OCT4. Moreover, phosphorylation of OCT4 induced binding of FBXW8, which reduced OCT4 protein stability and enhanced its proteasomal degradation. We also found that the mutant OCT4 (S347A) might delay the differentiation process of mouse ESCs and enhance the efficiency of generating iPSCs. These results demonstrated that OCT4 phosphorylation on serine 347 by JNKs plays an important role in its stability, transcriptional activities, and self-renewal of mouse ESCs.

INTRODUCTION

Embryonic stem cells (ESCs) are derived from the inner cell mass of the preimplantation blastocyst (Evans and Kaufman, 1981). Pluripotency, which is defined as the ability of a cell to differentiate into other cell types, is maintained during ESC self-renewal through the prevention of differentiation and the promotion of proliferation (Smith, 2001). The pluripotency of ESCs is maintained through an extensive transcriptional network (Niwa, 2007), and ESCs are capable of differentiation to multiple cell types induced by several transcription factors (Reubinoff et al., 2000). However, the transcriptional networks and molecular mechanisms that regulate the self-renewal and differentiation of ESCs are still unclear.

The POU transcription factor OCT4 (also known as OCT3/4, encoded by *POU5F1*) is one of the key transcriptional regulators involved in maintaining the pluripotency of self-renewing ESCs (Niwa, 2007) and in somatic cell reprogramming (Takahashi and Yamanaka, 2006; Yamanaka, 2009). *Oct4* expression is necessary for establishing the inner cell mass of the blastocyst (Nichols et al., 1998), and a precise amount of *Oct4* expression is critical for maintenance of pluripotency (Niwa et al., 2000). Recent studies show that post-translational modifications (PTMs), including sumoylation, ubiquitination, and phosphorylation, are important mechanisms in controlling the function of OCT4 (Cai et al., 2012; Lin et al., 2012; Swaney et al., 2009; Xu et al., 2004). For example, studies show that OCT4 can be sumoylated at lysine 118, which is located at the end of the N-terminal transactivation domain and next to the POU DNA binding domain. This

sumoylation of OCT4 significantly increases its stability, DNA binding ability, and transactivation function (Wei et al., 2007; Zhang et al., 2007). OCT4 can be ubiquitinated by WWP2, a HECT-type E3 ligase, to enhance its instability in mouse ESCs (Xu et al., 2004, 2009). Human OCT4A is phosphorylated at serine 111 by ERK1/2, which increases its ubiquitination and degradation (Spelat et al., 2012). OCT4 can also be phosphorylated by protein kinase A at serine 229 within its POU domain. The serine 229 phosphorylation of OCT4 sterically hinders both its ability to bind DNA and form a homodimer assembly (Saxe et al., 2009). These findings indicate that the site-specific phosphorylation of OCT4 can have different effects on its function. However, the specific signaling pathways mediating the PTMs of OCT4 and how these modifications affect specific gene transcription profiles remain poorly understood.

The c-Jun N-terminal kinases (JNKs) are members of the mitogen-activated protein kinase (MAPK) family of signaling proteins, including the extracellular signal-regulated kinases (ERKs) and the p38 MAP kinases. JNKs are encoded by two ubiquitously expressed genes (*Jnk1* and *Jnk2*) and by a third gene (*Jnk3*) that is largely restricted to brain, heart, and testis (Davis, 2000). Self-renewal or the maintenance of pluripotency of ESCs is enhanced by inhibition of the MAPK pathways (Binetruy et al., 2007). The role of these pathways in phosphorylation and activation of transcription factors has been extensively studied (Lee et al., 2006; Tiwari et al., 2012; Van Hoof et al., 2009). Studies using gene ablation of *Jnks* show that *Jnks* are required for the regulation of activator protein 1-dependent gene expression (Ventura et al., 2003) and that they play a critical role in the normal function of ESCs (Tiwari et al., 2012;

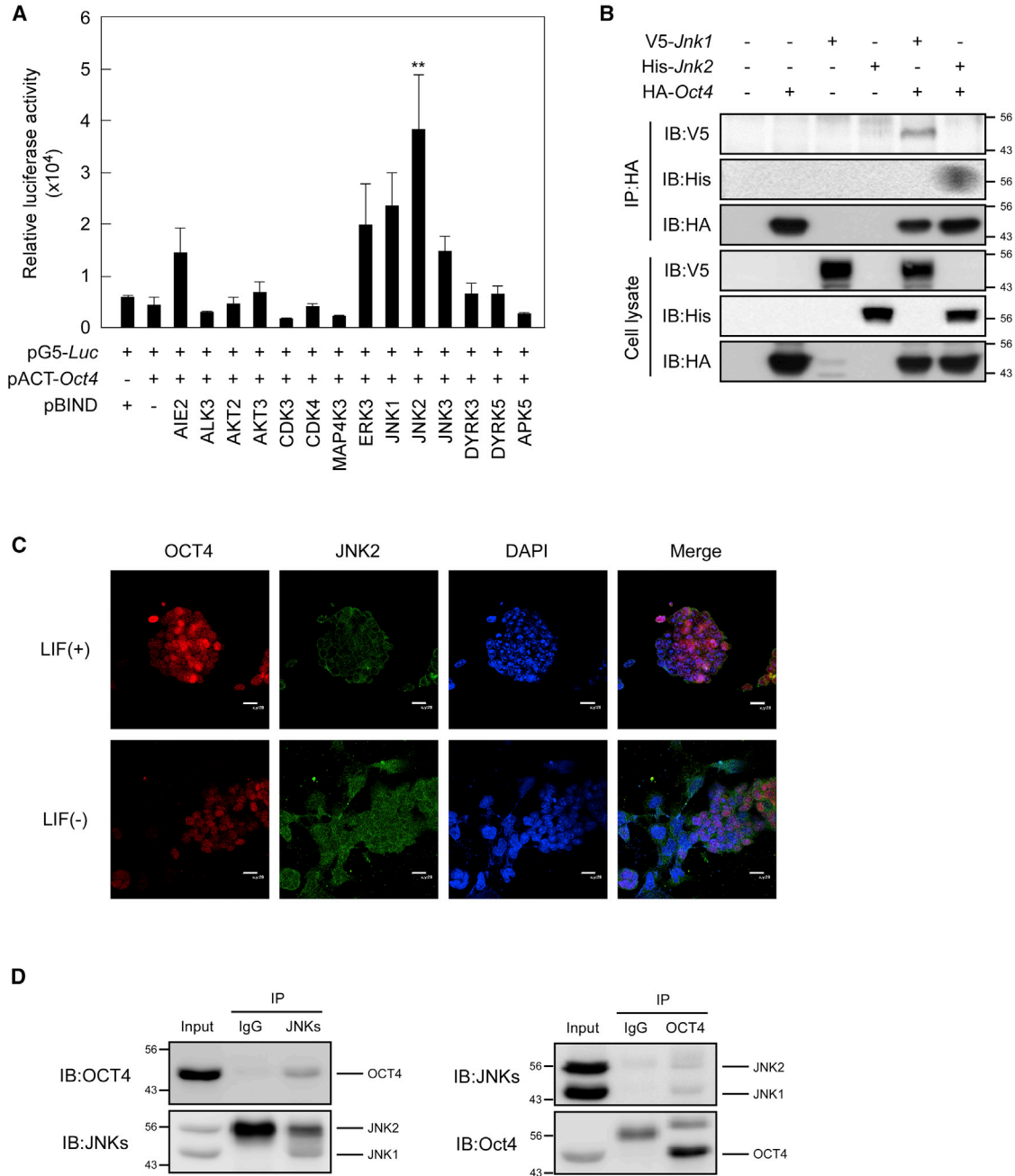


Figure 1. OCT4 Interacts with JNK1/2

(A) Assessment of the interaction of OCT4 with various kinases using *pACT-Oct4* and pBIND-protein kinases and the mammalian two-hybrid assay. Activity is indicated as relative luminescence units normalized to a negative control (value for cells transfected with only *pG5-luc/pACT-Oct4* = 1.0). Data are shown as mean \pm SD of values obtained from three independent experiments, and significant differences were evaluated using Student's *t* test (***p* < 0.001).

(B) Confirmation of OCT4 and JNK1/2 binding. The *pcMV-HA-Oct4* construct was co-transfected with *pcDNA3-V5-JNK1* or *pcDNA4-His-JNK2* into 293T cells to confirm the binding of OCT4 and JNK1/2. After culturing for 36 hr, cell lysates were immunoprecipitated (IP) with an HA monoclonal antibody. The co-immunoprecipitated JNK1/2 proteins were detected by western blot (IB) using anti-HRP-conjugated V5 and anti-His.

(C) Intracellular localization of OCT4 and JNK2 was detected by immunofluorescence staining. Dual staining of OCT4 and JNK2 indicated that OCT4-positive cells were co-stained with JNK2 under an LIF-supplemented (+) or -withdrawal condition (-). DAPI was used as the nuclear counterstain. Scale bars, 20 μ m.

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Xu and Davis, 2010; Yao et al., 2014). In this study, we demonstrate that JNKs interact with and phosphorylate OCT4. We reveal that this phosphorylation inhibits OCT4 transcriptional activity and stability. We also found that F-box protein 8 (FBXW8) can interact with the OCT4 protein phosphorylated at serine 347. This interaction enhances the ubiquitin (Ub)-proteasome pathway and, hence, the degradation of OCT4. Our data thus suggest that phosphorylation of serine 347 regulates OCT4 protein stability and proteasomal degradation in mouse ESCs.

RESULTS

OCT4 Is a Substrate of JNK1 and JNK2

Recent studies indicated that PTMs regulate the function of transcription factors, including *Sox2*, *Klf4*, and *Oct4* (Cai et al., 2012). Therefore, we hypothesized that OCT4 activity might be regulated by PTMs, especially phosphorylation. To identify the potential kinases of OCT4, we conducted a mammalian two-hybrid assay and found that JNK1 and JNK2 showed the highest binding activity with OCT4 (Figure 1A). To confirm the binding of OCT4 and JNKs, we co-transfected plasmids encoding *V5-Jnk1* or *His-Jnk2* with *HA-Oct4* into 293T cells. Results indicated that *HA-Oct4* was co-immunoprecipitated by *V5-Jnk1* or *His-Jnk2* (Figure 1B). To confirm whether OCT4 could directly interact with JNKs in mouse ESCs, we conducted immunofluorescence and endogenous immunoprecipitation assays. Immunofluorescence results showed that endogenous OCT4 co-localized with JNKs in the nucleus of mouse ESCs (Figures 1C and S1). To determine whether endogenous OCT4 and JNKs exist in the same protein complex, we performed co-immunoprecipitation experiments with cell extracts from E14Tg2a cells, and we found an interaction between endogenous OCT4 and JNKs. Endogenous OCT4 was detected in the JNK antibody-precipitated protein complexes (Figure 1D, left panel). Endogenous JNKs also were detected in OCT4 antibody-precipitated protein complexes, but not with the control immunoglobulin G (IgG) antibody (Figure 1D, right panel). These results demonstrate that the interaction between JNKs and OCT4 is observed in overexpression studies and possibly may occur in unmanipulated ESCs.

OCT4 Is Phosphorylated by JNKs on Serine 347

To identify the OCT4 domain that is involved in the interaction with JNK2, we constructed *pCMV-HA-Oct4* trun-

cated mutants (Figure 2A, upper panels). Deletion mutants of *Oct4* were individually transfected into 293T cells with *pcDNA4-His-Jnk2*, and expression was confirmed. Immunoprecipitation results using a His monoclonal antibody showed that JNK2 was co-immunoprecipitated with the full-length OCT4 and the OCT4 deletion mutants B and C (amino acids [aa] 117–268 and aa261–352; Figure 2A, lower panels). These results suggested that the COOH-terminal of OCT4 is important for binding with JNK2. Next, we determined whether OCT4 could be phosphorylated by JNKs. To identify the region of OCT4 that harbors potential phosphorylation sites for JNKs, we constructed a series of glutathione S-transferase (GST)-OCT4 deletion fragments (Figure 2B, upper panels). These proteins were expressed and purified as described in Experimental Procedures. To determine whether OCT4 is a selective substrate of JNK2, we conducted an *in vitro* kinase assay with [γ -³²P]ATP, active JNK2, and GST-OCT4 (full-length and deletion mutants). Autoradiography results showed that JNK2 phosphorylated both the OCT4 (full-length) and OCT4 deletion mutant C (aa261–352) (Figure 2B, lower panels). Therefore, we used mass spectrometry to identify the OCT4 sites that are phosphorylated by JNKs in the region spanning amino acids 261–352. The results indicated that JNK2 phosphorylated OCT4 on serine 347 (Ser347) (Figure 2C). The Ser347 site is also phosphorylated by JNK1 (Figures S2A and S2B). To confirm this phosphorylation site, we constructed mutants of GST-OCT4(261–352), substituting Ser347 for alanine (S347A). The proteins were purified and then directly subjected to an *in vitro* kinase assay with [γ -³²P]ATP and active JNK2. The result showed that phosphorylation by JNK2 was decreased in the mutant OCT4(261–352) (Figure 2D), demonstrating that Ser347 is the putative amino acid target for phosphorylation of OCT4 by JNKs (see also Figure S2C). The Ser347 site is highly conserved in OCT4 proteins from all species examined, suggesting that it is important for OCT4 function (Figure S2D).

JNKs Signaling Negatively Regulates OCT4 Transcription Activity

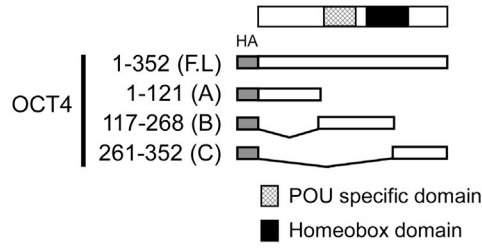
Mouse ESCs can be maintained in a self-renewal state with the addition of the recombinant leukemia inhibitory factor (LIF) to culture media. The role of the LIF-gp130-Stat3 axis in promoting ESC proliferation has been well defined (Niwa, 2001; Williams et al., 1988). We analyzed the expression level of OCT4 and phosphorylated JNKs

(D) Interaction of endogenous OCT4 and JNK1/2 in mouse ESCs grown under self-renewal conditions. Cell lysates were subjected to immunoprecipitation using mouse IgG, OCT4, or JNK antibodies, followed by western blot analysis using antibodies to detect JNKs and OCT4 as indicated.

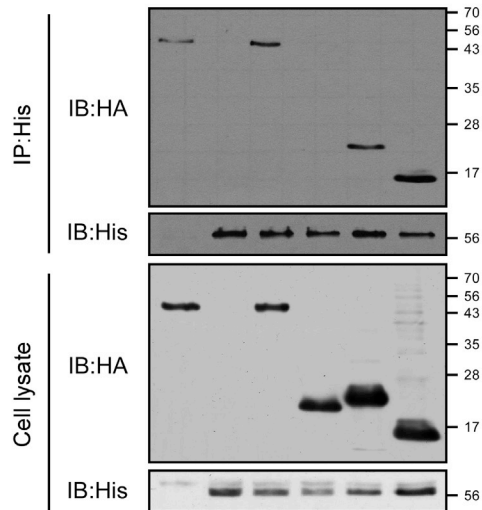
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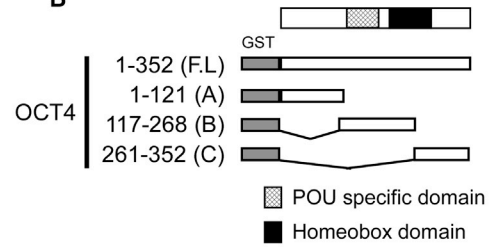
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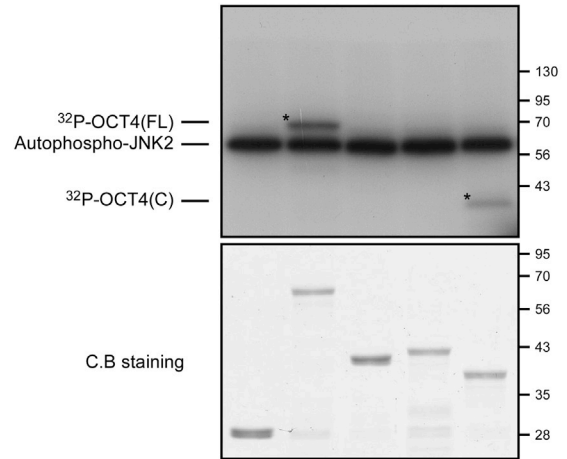
HA-Oct4(F.L.)	+	-	+	-	-	-
HA-Oct4(A)	-	-	-	+	-	-
HA-Oct4(B)	-	-	-	-	+	-
HA-Oct4(C)	-	-	-	-	-	+
His-Jnk2	-	+	+	+	+	+
HA-mock	-	+	-	-	-	-
His-mock	+	-	-	-	-	-



B



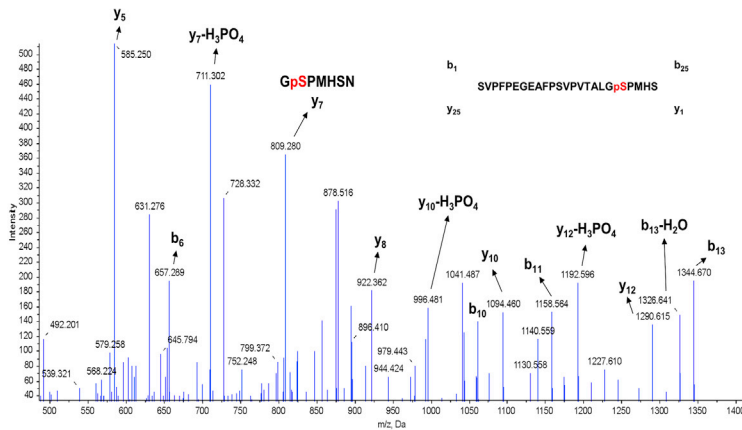
Active JNK2	+	+	+	+	+
GST-mock	+	-	-	-	-
GST-OCT4(F.L.)	-	+	-	-	-
GST-OCT4(A)	-	-	+	-	-
GST-OCT4(B)	-	-	-	+	-
GST-OCT4(C)	-	-	-	-	+



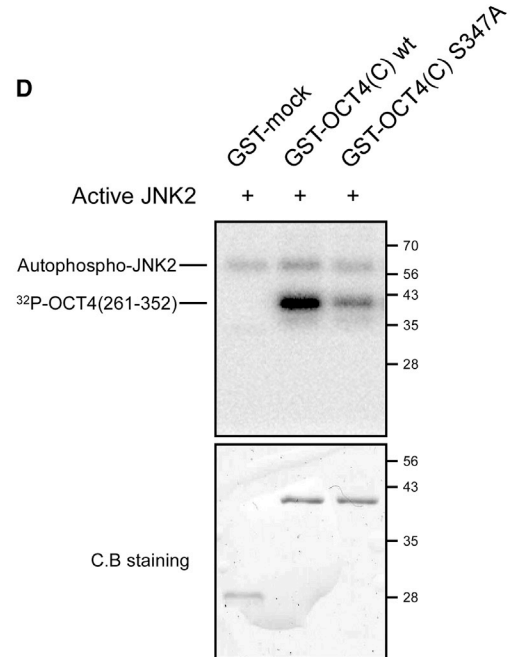
C

Conf. ¹	Sc ²	Prec m/z ³	z ⁴	Sequence	Theor MW ⁵	ΔMass ⁶	Site
99	17	878.7943	3	SVPFPEGEAFPSVPVTLGpSPMHSN	2633.1873	0.1738	S347

1, the confidence for the peptide identification; 2, the score for the peptide; 3, precursor m/z; 4, the charge for the fragmented ion; 5, theoretical precursor molecular weight for peptide sequence; 6, the difference between theoretical MW and experimental MW of the matching peptide sequence



D



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in mouse ESCs grown under the self-renewal (LIF+) or differentiation (LIF-) culture condition. The expression of OCT4 was gradually decreased and JNK phosphorylation was increased under differentiation conditions, suggesting that LIF successfully sustains OCT4 expression levels to maintain self-renewal (Figure S3A). Recent studies showed that the self-renewal ability of ESCs is also enhanced by inhibition of the MAPK signaling pathway (Yao et al., 2014; Ying et al., 2008). To examine the relationship between OCT4 and JNK inhibition in the differentiation process, we induced differentiation of ESCs by withdrawing LIF and inhibiting JNK activation by adding SP600125. We confirmed that inhibition of the JNK signaling pathway increased the number of alkaline phosphatase (AP)-positive colonies in the differentiation process (Figure 3A). In comparison with the MAPK inhibitor, a higher number of undifferentiated colonies were detected in the SP600125-treated group compared with the other inhibitor-treated groups (Figure S3B). The total protein levels of OCT4 were restored (Figure 3B) and the phosphorylated serine signal of OCT4 was decreased (Figure S3C) by the addition of SP600125 in the differentiation of ESCs. This implies that inhibition of JNK activation by SP600125 treatment significantly increases the self-renewal of mouse ESCs. We also hypothesized that the site-specific phosphorylation of OCT4 can have different effects on its function. To determine the effect of OCT4 phosphorylation by JNKs on the transcriptional activity of OCT4, we measured luciferase activity in HeLa cells or E14Tg2a cells that were co-transfected with the *phOct4-luciferase* reporter, *pCMV-HA-Oct4* (wild-type or S347A mutant) and different doses of *pcDNA4-His-Jnk2*. *Oct4* promoted *phOct4-luciferase* activity and the high concentration of *Jnk2* decreased reporter gene activity promoted by *Oct4* (Figure 3C). Mutation of *Oct4* at Ser347 recovered the suppression of transcriptional activity induced by *Jnk2* in HeLa cells (Figure 3D) or E14Tg2a

cells (Figure 3E). These results indicated that phosphorylation of OCT4 at Ser347 negatively regulates OCT4 transcriptional and transactivation activities, suggesting that the JNK-OCT4 signaling axis might negatively regulate self-renewal activities during ESC maintenance.

Inhibition of OCT4 Ser347 Phosphorylation Delays Mouse ESC Differentiation Induced by Withdrawal of LIF

To determine the effects of OCT4 Ser347 phosphorylation on the self-renewal activity of ESCs, we created OCT4 (wild-type or S347A mutant)-overexpressing stable cells by electroporation. We induced differentiation of overexpressing stable or control E14Tg2a cells by LIF withdrawal and examined cell morphology using AP staining, then confirmed the level of hemagglutinin (HA)-tagged OCT4 proteins. The results show that both OCT4 wild-type- and OCT4 mutant (S347A)-overexpressing stable cells enhanced the number of AP-positive colonies compared with control E14Tg2a cells. In particular, the OCT4 mutant-expressing stable cells delayed the differentiation of ESCs (Figure S4A). The expression of endogenous OCT4 and SOX2 was gradually decreased over 4–6 days after withdrawal of LIF, but the expression level of the HA-tagged OCT4 mutant was maintained in the differentiation process (Figure S4B). To diminish endogenous OCT4 expression, we used lentiviruses encoding short hairpin RNAs (shRNAs) against OCT4 (Figure 4A), after which we introduced HA-tagged OCT4 (wild-type or S347A mutant) into endogenous OCT4 knockdown cells (Figure 4B). We induced differentiation of those cells by LIF withdrawal and examined cell morphology using AP staining (Figure 4C). The results show that the cells expressing the OCT4 mutant (S347A) delayed the differentiation process. To evaluate the expression level of the HA-tagged OCT4 protein, we performed immunofluorescence analysis with HA and markers of undifferentiated cells (NANOG,

Figure 2. OCT4 Is Phosphorylated by JNK2 on Serine 347

(A) Structure and schematic diagrams of HA-tagged *Oct4* deletion mutant constructs (upper panels). Determination of binding between HA-tagged *Oct4* deletion mutants and His-tagged *Jnk2*. OCT4 co-immunoprecipitated wild-type and deletion mutants were detected by western blot using an HRP-conjugated HA antibody (lower panels).

(B) Identification of the OCT4 domain that is phosphorylated by JNK2. Each GST-truncated deletion mutant was constructed and the GST fusion proteins purified (upper panels). The proteins were directly subjected to an *in vitro* kinase assay with [γ - 32 P]ATP and active JNK2. The phosphorylated GST-OCT4 proteins were visualized by autoradiography and the purified GST fusion proteins were stained with Coomassie blue as a control. The asterisks indicate phosphorylation (lower panels).

(C) Tandem mass spectrometry spectra of the OCT4 site phosphorylated by JNK2. OCT4 is phosphorylated at the Ser347 residue by JNK2. The precursor mass (m/z) of 878.7943 was matched to the triply charged peptide, SVPFPEGEAFSPVPTALGSPMHSN, indicating that Ser347 was phosphorylated. The y_7 ion at 809.28 indicates that Ser374 is phosphorylated. The presence of b_6 , b_{13} , y_5 , and y_{12} show non-phosphorylated Ser228, Ser339, Thr343, and Ser351 residues, respectively.

(D) *In vitro* kinase assay of GST-OCT4₂₆₁₋₃₅₂-S347A using [γ - 32 P]ATP and active JNK2. The results were visualized by autoradiography and the purified GST fusion proteins were stained with Coomassie blue as a control.

See also Figure S2.

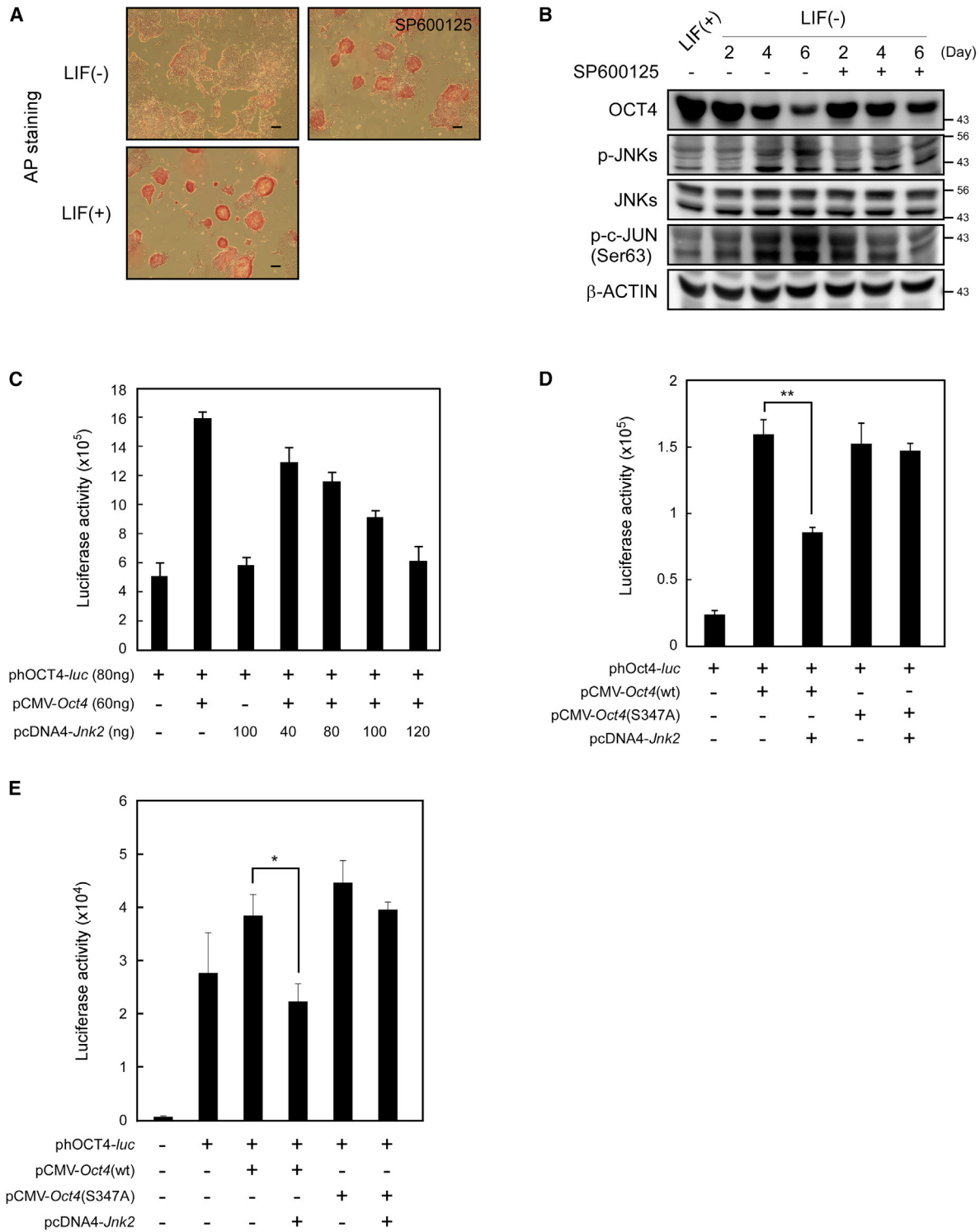


Figure 3. The Transcription Activity of OCT4 Is Inhibited by JNK2

(A) Inhibition of JNK signaling by SP600125 suppresses differentiation of mouse ESCs. Characterization of mouse ESCs cultured for 5 days in the presence of SP600125 (1 μ M) or vehicle (0.125% DMSO) upon withdrawal of leukemia inhibitory factor (LIF). Scale bars, 100 μ m. (B) Protein levels of OCT4 and phosphorylated c-JUN in mouse ESCs under differentiation conditions with or without SP600125. The protein levels of OCT4 and the indicated gene products were analyzed by western blotting.

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OCT4, and SOX2; Figures 4D and S4C). These results indicated that the OCT4 mutant (S347A) is resistant to the differentiation process induced by LIF withdrawal. This suggests that sustained expression of the OCT4 mutant might delay the differentiation of mouse ESCs.

The OCT4 S347A Mutant Enhances Protein Stability by Blocking Ubiquitin-Mediated Proteolysis

Previous studies reported that the OCT4 protein expression levels are critical for maintenance of pluripotency and self-renewal in ESCs (Niwa et al., 2000), and that a proteasome-dependent mechanism plays a key role in the regulation of OCT4 protein levels (Saxe et al., 2009; Xu et al., 2009). Based on recent studies, we expected that the JNK-mediated phosphorylation of OCT4 would regulate its intracellular stability. To examine whether OCT4 stability is regulated by proteasome degradation, we treated mouse ESCs with cycloheximide (CHX) to block the synthesis of new proteins. Our data revealed that the endogenous OCT4 protein levels in mouse ESCs were gradually decreased by CHX treatment, whereas pretreatment with the proteasome inhibitor, MG132, attenuated OCT4 degradation, indicating that the degradation requires proteasome activity (Figure 5A). To determine the relationship between JNK-mediated phosphorylation of OCT4 at Ser347 and OCT4 stability, an HA-tagged OCT4 wild-type and mutant S347A construct was each expressed in 293T cells, and the cells were treated with CHX for up to 6 hr. Western blot analysis showed that the OCT4 wild-type protein degraded faster than the OCT4 mutant (Figure 5B). This indicates that the phosphorylation of OCT4 at Ser347 reduces OCT4 protein stability. To confirm whether this phenomenon could be due to the blockade of ubiquitin-mediated proteolysis, we performed an *ex vivo* ubiquitination assay by transfecting 293T cells with FLAG-tagged ubiquitin together with expression vectors as indicated. Our results showed that ubiquitination of OCT4 was increased by co-transfection with JNK1 or JNK2 (Figure 5C), but inhibition of OCT4 phosphorylation by mutation of serine to alanine decreased OCT4 ubiquitination (Figure 5D). These results demonstrate that OCT4 protein stability might be regulated by a JNK-mediated phosphorylation-dependent mechanism and that phosphorylation of OCT4 at Ser347 enhances its ubiquitin-mediated proteolysis.

FBXW8 Binds OCT4 and Regulates Its Protein Stability

The ubiquitin-proteasome system plays an important role in the control of protein stability and cellular signaling. The F-box proteins are substrate-recognition components responsible for the substrate specificity among the E3 ubiquitin ligases (Hershko and Ciechanover, 1998; Ho et al., 2006). To identify the E3 ligase responsible for targeting OCT4, we screened several F-box E3 ligases using immunoprecipitation. Notably, we found that FBXW4 and FBXW8 were co-immunoprecipitated with OCT4 (Figure S5A). To investigate the possible effect of OCT4 phosphorylation on its degradation by FBXW4 or FBXW8, we performed immunoprecipitation analysis of the interaction between FBXWs and OCT4 (wild-type or mutant). In transfected 293T cells, wild-type OCT4 interacted with FBXW4 or FBXW8, whereas its binding affinity was only abolished in FBXW8 by the S347A mutation (Figures S5B and S5C). These results demonstrated that OCT4 interacts with FBXW8 and that phosphorylation of Oct4 on Ser347 is essential for its ubiquitination by FBXW8.

Phosphorylation of OCT4 Plays a Crucial Role in Somatic Cell Reprogramming

Induced pluripotent stem cells (iPSCs) can be generated directly from somatic cells by ectopic expression of the transcription factors *Oct4*, *Sox2*, and *Klf4* in combination with *c-Myc* (OSKM) (Takahashi and Yamanaka, 2006). To examine the effects of OCT4 phosphorylation at Ser347 on the generation of iPSCs, we infected SKH-1 mouse embryonic fibroblasts with retroviruses carrying OSKM. On day 2 after infection, the cells were replated onto an inactivated feeder layer in embryonic stem medium supplemented with LIF. Within 10–12 days after infection, we identified colonies resembling ESCs. At 3 weeks after infection, we counted the number of iPSC colonies that were AP positive and had ESC-like morphology of a round shape and distinct edge. We found that mutant O(S347A) SKM-infected MEFs generated iPSCs more efficiently than wild-type O(wt) SKM-infected MEFs (Figure 6A). We also determined the generation of iPSCs using mouse tail tip fibroblasts (TTFs). Our results shown that O(S347A) SKM-infected TTFs generated iPSCs more efficiently than wild-type O(wt) SKM-infected TTFs (Figure S6). To characterize our iPSCs in detail, we randomly picked several clones and then confirmed the expression of ESC pluripotency

(C) JNK2 negatively regulates OCT4 transcription activity. The *phOct4-luciferase* reporter plasmid and the *pCMV-Oct4* plasmid were co-transfected with different doses of the *pcDNA4-Jnk2* plasmid into HeLa cells.

(D and E) Effect of *Oct4* mutation on *Jnk2*-mediated suppression of *Oct4* transcription activity. The *phOct4-luciferase* reporter plasmid or *pCMV-Oct4* (wild-type or S347A mutant) plasmid was co-transfected with the *pcDNA4-Jnk2* plasmid into HeLa cells (D) or E14Tg2a cells (E). For (C) to (E), the luciferase activity was measured at 36 hr after transfection and normalized against *Renilla* luciferase activity, and data are shown as mean \pm SD of values obtained from three independent experiments; significant differences were evaluated using Student's *t* test (**p* < 0.05, ***p* < 0.001). See also Figure S3.

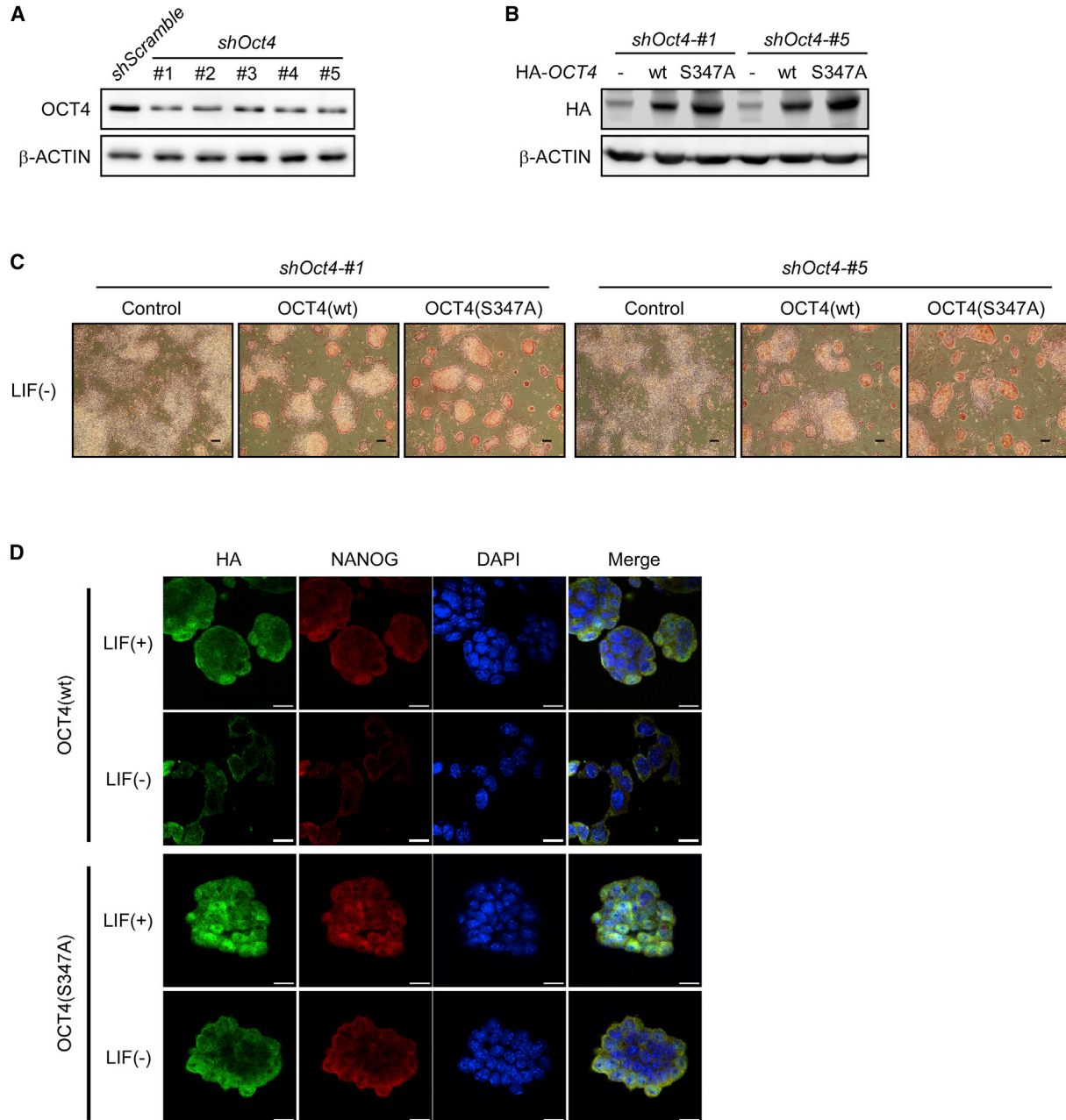


Figure 4. The OCT4 Mutant Protein Delays the Differentiation Process of Mouse ESCs

(A) shRNA-mediated knockdown of OCT4 in E14Tg2a cells.

(B) The exogenous HA-tagged OCT4 (wild-type or S347A mutant) proteins were visualized by western blotting with anti-HA in endogenous OCT4 knockdown E14Tg2a cells.

(C) HA-tagged OCT4 (wild-type or S347A mutant)-expressing cells were cultured without LIF for 5 days. Cells were fixed and stained using an alkaline phosphatase (AP) staining kit. Scale bars, 100 μ m.

(D) Immunofluorescence staining of HA and NANOG in E14Tg2a ESCs stably expressing wild-type or mutant OCT4 under a LIF-supplemented (LIF+) or -withdrawal (LIF-) condition. DAPI was used as the nuclear counterstain. Scale bars, 20 μ m.

See also [Figure S4](#).

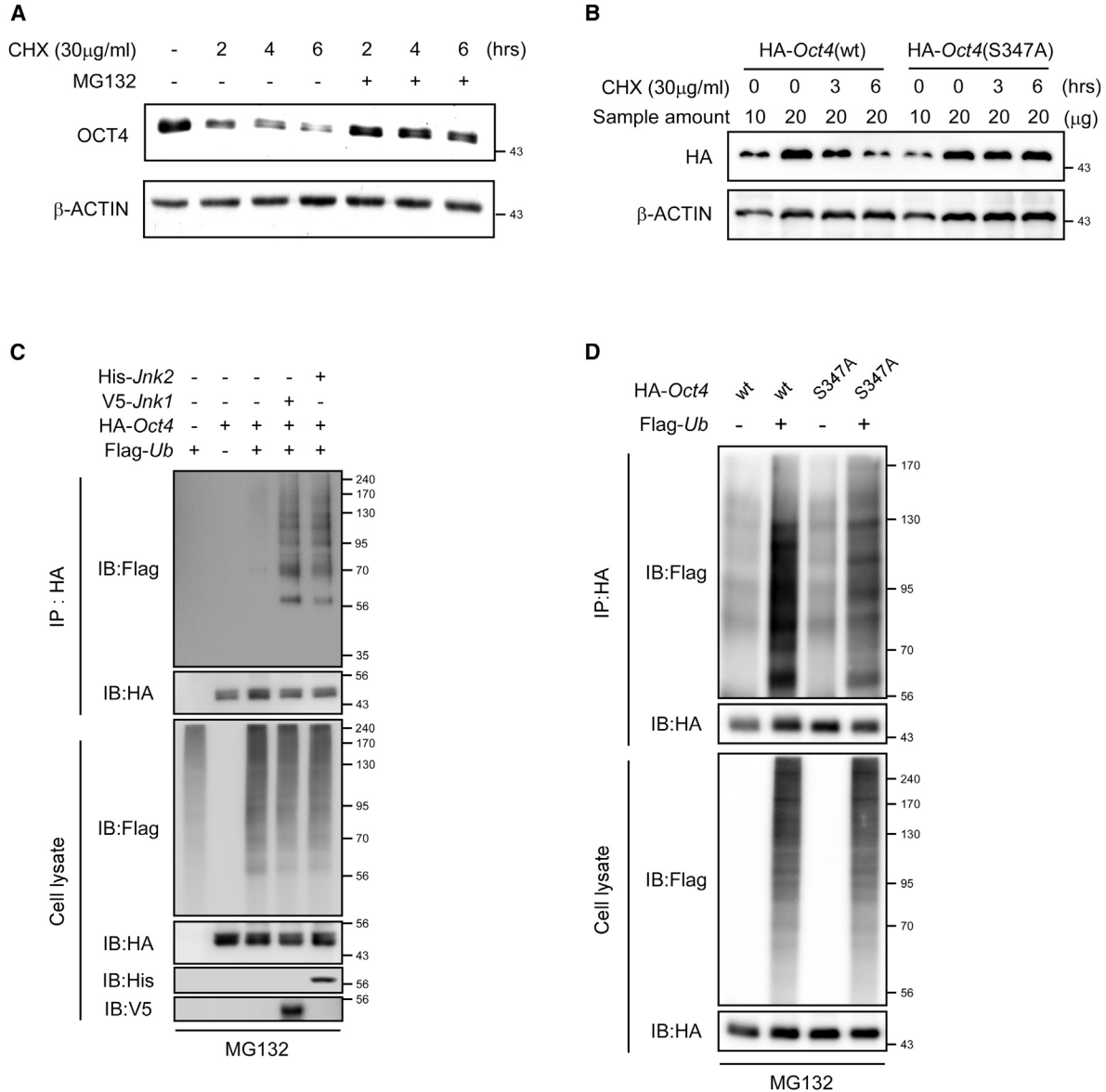


Figure 5. OCT4 Protein Stability Is Modulated by Ubiquitin-Mediated Proteasomal Degradation

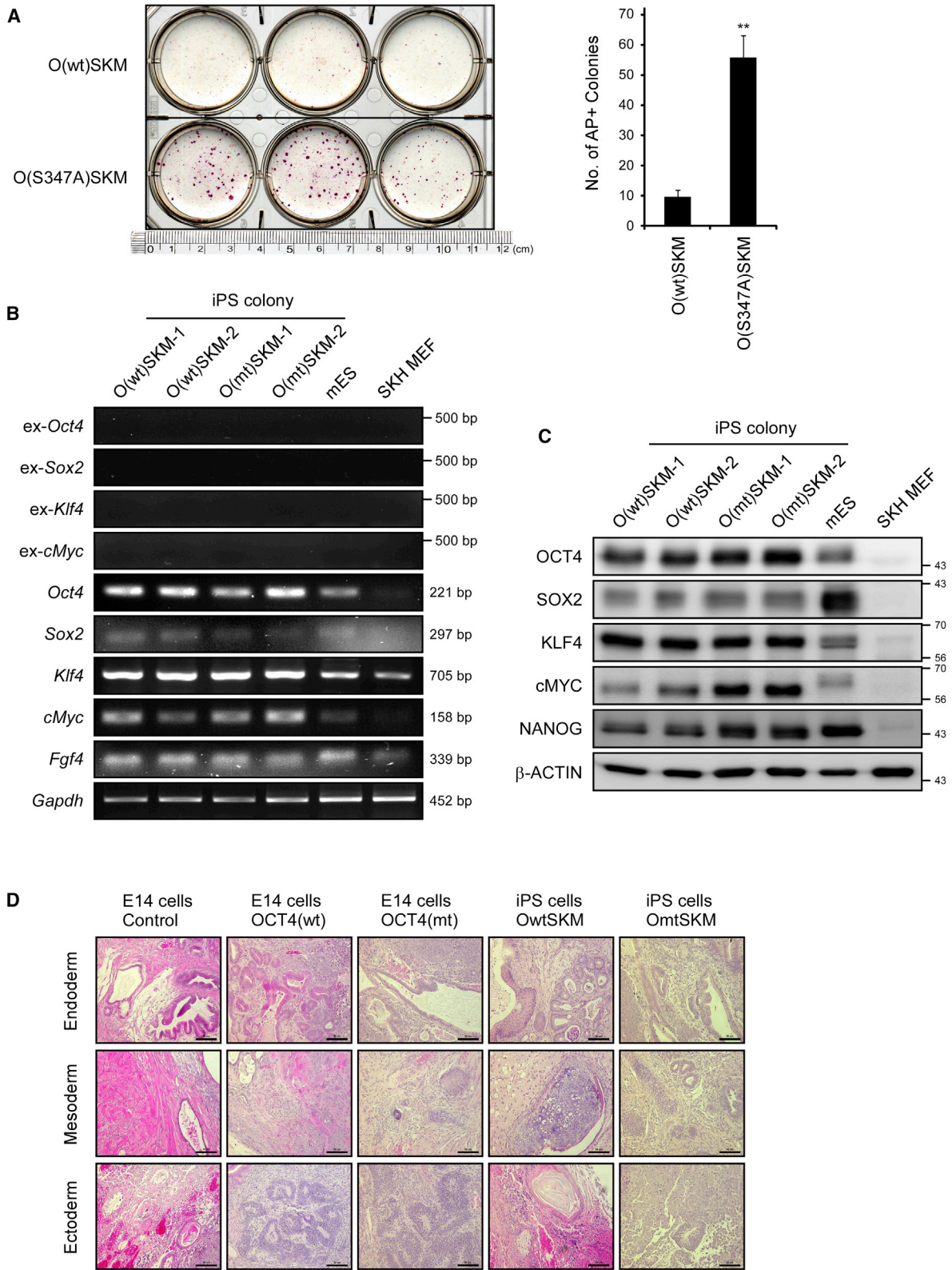
(A) OCT4 protein levels are regulated through the proteasomal degradation pathway. E14Tg2a cells were treated with cycloheximide (CHX) in combination with or without MG132 (20 μM), a proteasome inhibitor. The proteins were extracted at the indicated time point and then subjected to western blot analysis.

(B) The S347A mutation impairs OCT4 degradation. 293T cells were transfected with HA-tagged *Oct4* (wild-type or S347A mutant) and treated with CHX for the indicated time periods. Whole-cell lysates were immunoblotted with the indicated antibodies.

(C) JNK1/2 induces ubiquitination of exogenous OCT4. The indicated combination of expression vectors was co-transfected into 293T cells and cells were cultured for 36 hr. Cells were treated with MG132 (20 μM) for 4 hr before cells were harvested. The ubiquitination of OCT4 was visualized by immunoprecipitation (IP) with anti-HA and immunoblotted with anti-FLAG.

(D) The S347A mutation inhibits ubiquitination of OCT4. The 293T cells were transfected with the indicated combination of expression vectors and cultured for 36 hr. MG132 (20 μM) was added 4 hr before cell harvesting. The ubiquitination of OCT4 was visualized by immunoprecipitation with anti-HA and immunoblotted with anti-FLAG.

See also [Figure S5](#).



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markers by semi-quantitative RT-PCR analysis or western blotting. Results showed that the established iPSC clones at passage 5 underwent retroviral silencing and expressed endogenous ESC markers at levels that were similar to those of mouse ESCs (Figures 6B and 6C). Moreover, we verified the *in vivo* developmental capacity of iPSCs or OCT4 (wild-type or S347A mutant)-overexpressing stable cells by teratoma formation. Results showed that every cell group showed a similar *in vivo* differentiation capacity because they formed cells from all three germ layers (Figure 6D). These results demonstrate that inhibition of OCT4 phosphorylation on Ser347 can improve the reprogramming efficiency of SKH-1 MEFs transduced by the four-factor OSKM without affecting the maintenance of iPSCs after exogenous transgenes have been silenced.

DISCUSSION

OCT4 is an essential factor of the core transcriptional regulatory network for ESCs and for reprogramming somatic cells. Previous studies reported that maintenance of the pluripotent state is controlled by the expression level of OCT4 *in vitro* (Radzishheuskaya et al., 2013). Inactivation of OCT4 in mouse ESCs induced the loss of self-renewal activity and the formation of the trophectoderm, whereas 2-fold induction of OCT4 triggers differentiation into endoderm and mesoderm (Ivanova et al., 2006; Niwa et al., 2000). Therefore, the maintenance and induction of pluripotency require precise OCT4 protein levels, and a change in OCT4 levels directs different cell fates. Recent reports indicate that PTMs are important complementary mechanisms for OCT4 function (Cai et al., 2012; Lin et al., 2012; Saxe et al., 2009; Spelat et al., 2012; Swaney et al., 2009; Wei et al., 2007; Xu et al., 2004, 2009; Zhang et al., 2007).

Phosphorylation is one of the most common protein modifications that occurs as a mechanism to regulate the biological activity of a protein. Several large-scale proteomic studies have identified a global pattern of protein phosphorylation dynamics in pluripotent cells (Brumbaugh

et al., 2012; Saxe et al., 2009; Swaney et al., 2009; Van Hoof et al., 2009). Although OCT4 transcriptional regulation has been extensively studied, its specific phosphorylation sites and regulatory mechanisms have not been fully determined. Therefore, predicting kinase-substrate relationships might support the further understanding of phosphorylation-based mechanisms governing pluripotency and self-renewal of ESCs. In this study, we used the mammalian two-hybrid assay to screen potential kinases that might affect the function of OCT4. In particular, we focused on the role of JNKs because they have been shown to regulate the pluripotency of ESCs (Xu and Davis, 2010; Yao et al., 2014). Our study identified Ser347 as a phosphorylation site on OCT4 that could be phosphorylated by JNKs. We determined that JNK-mediated OCT4 phosphorylation reduces its transcriptional activity, protein stability, and self-renewal ability of mouse ESCs. In addition, blockade of Oct4 phosphorylation on Ser347 aids in OSKM-mediated iPSC reprogramming from SKH-1 MEFs or mouse TTFs. Our findings provide evidence showing that the site-specific phosphorylation of OCT4 can have different effects on its stability and transcriptional activities and could also potentially explain why inactivation of JNKs promotes reprogramming of somatic cells (Huang et al., 2009).

In many circumstances, the OCT4 protein is synthesized and then degraded. One of the major mechanisms for the degradation of intracellular proteins is the Ub-proteasome pathway. Conjugation of Ub to substrates can be a polyubiquitination with a polymeric Ub chain. In this mechanism, E3 ligase families perform the final step in the ubiquitination cascade, catalyzing transfer of Ub from the E2 enzyme to form a covalent bond with a substrate lysine (Hershko and Ciechanover, 1998). F-box proteins are substrate-recognition components among the families of E3 ubiquitin ligases, and we found that FBXW8 is the partner most responsible for OCT4 stability. FBXW8 reduced OCT4 protein stability, resulting in ESC differentiation. Further studies will be required to elucidate the detailed molecular mechanism of FBXW8 action on ESCs.

In conclusion, our data demonstrate that Ser347 phosphorylation of OCT4 by JNKs can reduce OCT4 protein

Figure 6. The S347A Mutation of OCT4 Enhances Reprogramming Efficiency

(A–C) Generation and characterization of iPSCs from SKH-1 mouse embryonic fibroblasts (MEFs). (A) Representative images of AP-positive iPSC colonies from MEFs transduced with 0(wt)SKM or 0(S347A) SKM (left panel). AP-positive colonies were counted under a light microscope. Data are shown as mean \pm SD from three independent experiments (**p < 0.001). (B) RT-PCR analysis verified the expression of ESC marker genes, endogenous genes, and lentiviral transgenes (exo) in iPSC clones. ESCs were used as a positive control and SKH-1 MEFs as a negative control. *Gapdh* was used as an internal control to verify equal amounts of cDNA. (C) iPSC colonies were picked and propagated under the same conditions as ESCs. Western blot analysis verified the expression of iPSC and ESC marker genes. (D) *In vivo* developmental potential of iPSCs assessed by teratoma assay. Cells were injected into athymic nude mice and H&E staining was performed. After tumors grew for 5–6 weeks, mice were euthanized and tissues harvested and fixed in 4% paraformaldehyde. Samples were embedded in paraffin and stained with H&E to assess the presence of tissues of the ectoderm, endoderm, and mesoderm. Scale bars, 50 μ m. See also Figure S6.



stability, its transcriptional activities, and the self-renewal ability of mouse ESCs. This phosphorylation modulates OCT4 protein degradation through an Ub-mediated proteasomal degradation by FBXW8. Therefore, this study provides one additional important step forward toward the understanding of how post-translational modification of OCT4 is involved in the pluripotency of ESCs and the reprogramming of somatic cells.

EXPERIMENTAL PROCEDURES

Cell Culture

Mouse ESCs (E14Tg2a) were maintained on MEFs, which were inactivated using γ -irradiation in standard complete ESC culture medium. The medium was supplemented with 15% heat-inactivated ESC qualified fetal bovine serum (FBS) (Millipore, Billerica, MA), 0.055 mM β -mercaptoethanol (Invitrogen), 2 mM L-glutamine, 0.1 mM minimum essential medium non-essential amino acids, and 1,000 U/mL LIF (Millipore). ESCs were passaged at 70% confluence on the feeder cells. HeLa and 293T cells were cultured in DMEM and 10% heat-inactivated FBS in a 37°C, 5% CO₂ incubator. Cells were maintained by medium change every other day and then split at 80% confluence.

Viral Preparation and Induction of Pluripotent Stem Cells

The Moloney-based (pMXs) retroviral vectors with the cDNAs for mouse *Oct4*, *Sox2*, *Klf4*, and *c-Myc* were co-transfected into 293T cells together with *pCI-GPZ* and *pCI-VSV-G* helper plasmids using the jetPEI transfection reagent (Qbiogene). Viral supernatant fractions were collected 48 hr later, filtered, and used directly for infection.

Mammalian Two-Hybrid Assay

The mammalian two-hybrid assay was conducted according to the manufacturer's protocols (Checkmate Mammalian Two-Hybrid System assay; Promega). In brief, HEK293 cells were seeded in 48-well plates at a density of 2×10^4 cells per well and cultured for 18 hr before transfection. Various plasmids including *pACT-Oct4*, *pBIND-kinases*, and *pG5-luciferase* were combined in the same molar ratio (1:1:1), and the total amount of DNA was not more than 100 ng/well. The transfections were performed using jetPEI following the manufacturer's protocols. For the luciferase assay, the cells were disrupted by luciferase lysis buffer and incubated for 30 min at room temperature with gentle shaking. The luciferase activity was measured by a computer-controlled luminometer (Luminoskan Ascent, MTX Lab Systems, McLean, VA) with the automatic addition of 100 μ L of substrate buffer. The relative firefly luciferase activity was calculated using the *pG5-luciferase* basal control and normalized against *Renilla* luciferase activity, which included the pBIND vector as an internal control.

Protein Extraction and Western Blot Analysis

Cells were harvested and disrupted with RIPA cell lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 1%

sodium deoxycholate, 0.1% SDS, and Complete Protease Inhibitor Cocktail Tablets [Roche, Indianapolis, IN]). The proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk, and target proteins were detected with specific antibodies and visualized by enhanced chemiluminescence.

Immunoprecipitation

Cell lysates in a total volume of 500 μ L were immunoprecipitated with 2 μ g of each respective antibody and 40 μ L of protein A/G Sepharose beads. The samples were rotated at 4°C overnight. After the beads were washed three times with lysis buffer, the pellets were analyzed by SDS-PAGE.

Preparation of GST Fusion Proteins and the *In Vitro* Kinase Assays

To generate GST fusion proteins, we amplified partial sequences for OCT4 amino acids 1–121, 117–268, and 261–352 from the full-length mouse *Oct4* cDNA and subcloned them into the pGEX-5XC vector. Site-directed mutagenesis was performed using the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The BL21 bacterial strain was used to express the GST-OCT4 fusion proteins, which were then purified. Wild-type GST-OCT4, truncated deletion, or point mutant proteins were used for an *in vitro* kinase assay with active JNK1 or JNK2 (Millipore). Reactions were conducted at 30°C for 30 min in a mixture containing 50 μ M unlabeled ATP and 10 μ M $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction was stopped by adding 6 \times SDS sample buffer, and samples were boiled and separated by 10% SDS-PAGE and visualized by autoradiography.

Enrichment of JNK1/2-Phosphorylated OCT4 Peptides

To identify phosphorylation sites on JNK-phosphorylated OCT4, we isolated OCT4 protein from *Oct4*- and *Jnk*-overexpressing cells. Only *Oct4*-overexpressing cells were used as a negative control. OCT4 protein was denatured by urea buffer (7 M urea, 2 M thio-urea, 2% CHAPS), reduced with 4 mM DTT for 1 hr at 37°C, and alkylated with 14 mM iodoacetamide (IAM) for 45 min at room temperature under dark conditions. Excess IAM was quenched with excess DTT to reach a final concentration of 7 mM. Subsequently, the sample was diluted with 50 mM ammonium bicarbonate to ensure less than 1 M of urea content, and digested at 37°C with chymotrypsin (Fisher Scientific) at an enzyme content of 2% (w/w) for 16–20 hr. These enzymatic peptides were dried by vacuum evaporation using a Speed-Vac. Phosphorylated peptides of OCT4 were enriched using the Titansphere phos-TiO Kit (GL Sciences) according to the manufacturer's instructions. The TiO₂ spin tip was equilibrated with buffer A (2% trifluoroacetic acid/acetone nitrile ratio of 1:4) and buffer B (100% lactic acid/buffer A ratio of 1:3) prior to loading of peptides. Peptides were loaded to the TiO₂ spin tip with buffer B. The spin tip was washed with buffer B and further washed with buffer A. Phosphopeptides were eluted using 50 μ L each of 5% ammonium hydroxide and 5% pyrrolidine. To remove lactic acid from the enriched phosphopeptides, we added 100 μ L of 20% formic acid to the eluents. The mixture was dried by vacuum evaporation and then cleaned up using Graphite Spin Columns (Thermo Scientific) according to the manufacturer's instructions.



Identification of Phosphorylation Sites on JNK1/2-Phosphorylated OCT4

The ABSciex TripleTOF 5600 system that was coupled with Eksigent 1D+ Nano LC system was used to identify phosphorylated sites of OCT4. Nano LC of enzymatic peptides was performed with the Eksigent 1D+ Nano LC equipped with the cHiPLC nanoflex system. Binary solvents A1 and B1 contained 0.1% formic acid in water and acetonitrile, respectively. Peptides were loaded onto the column and then eluted from the column with a linear gradient of 5%–40% binary solvent B1 for 30 min at a flow rate of 0.3 $\mu\text{L}/\text{min}$. Mass spectrometry analysis of enzymatic peptides was performed using the ABSciex TripleTOF 5600 system. For all measurements, the mass spectrometer was operated in positive-ion and high-sensitivity mode. All analyses were performed using the NanoSpray III source. The mass spectrometry was calibrated by acquisition of [Glu¹]fibrinopeptide (25 pmol/ μL). The raw data were processed and searched with ProteinPilot software (version 4.0) using the Paragon algorithm. Protein identification was obtained by searching the UniProtKB mouse database, and filtered at a $\geq 95\%$ confidence cutoff. Peptides for phosphorylated OCT4 were identified at a 1% global false discovery rate level.

Luciferase Reporter Gene Assay

HeLa cells were seeded in 48-well plates at a density of 1×10^4 cells per well. After 24 hr, the cells were transfected using jetPEI. Cells were harvested at 48 hr after transfection and analyzed for firefly luciferase activity. Transfection efficiency was normalized with a *Renilla* plasmid as an internal control.

Establishing Stable Transfectants of ESCs by Electroporation

E14Tg2a cells (2×10^7 cells) were electroporated with a linearized *pBabe-HA-Oct4* (wild-type or S347A mutant) plasmid (20 μg) at 230 V and 500 μF using the Gene Pulser X (Bio-Rad Laboratories, Hercules, CA). The cells were plated onto 60-mm dishes and puromycin (2 $\mu\text{g}/\text{mL}$; Sigma-Aldrich) selection was initiated at 2 days after electroporation. After selection on a medium containing puromycin, the resulting cell types were confirmed by immunoblot analysis with an HA antibody.

RNA Interference

The lentiviral expression vectors (*PLKO.1-shOct4*) and packaging vectors (pMD2.G and psPAX) were purchased from Genomics Center, University of Minnesota. The sense sequences were as follows: OCT4 shRNA#1: GCC GAC AAC AAT GAG AAC CTT; OCT4 shRNA#2: CCT ACA GCA GAT CAC TCA CAT; OCT4 shRNA#3: CGT TCT CTT TGG AAA GGT GTT; OCT4 shRNA#4: CAA TGC CGT GAA GTT GGA GAA; OCT4 shRNA#5: CAA GGG AGG TAG ACA AGA GAA.

Alkaline Phosphatase Staining and Immunofluorescence Staining

For colony staining, ESCs were cultured in the appropriate medium, fixed with 4% paraformaldehyde in PBS for 2 min at room temperature, after which AP staining was performed with the Alkaline Phosphatase Detection Kit (Millipore) following the man-

ufacturer's protocols. AP-positive colonies were observed under a light microscope (Olympus, Center Valley, PA). Immunofluorescence staining was performed using primary antibodies (all 1:200) to detect OCT4 (SC-5279, Santa Cruz Biotechnology), JNK1 (SC-1648, Santa Cruz), and JNK2 (SC-827, Santa Cruz). Nuclei were counterstained with DAPI.

Teratoma Assay and Histological Analyses

iPSCs were suspended in iPSC culture medium (3×10^7 cells/mL), and 100 μL of the cell suspension (3×10^6 cells/mL) was subcutaneously injected into the dorsal flank of athymic nude mice (6 weeks old). After 5 weeks the tumors were surgically dissected, fixed in 10% buffered formaldehyde, and embedded in paraffin. Paraffin-embedded sections were stained with H&E to determine cell types. Images were captured by a light microscope. All animal handling and procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Protein Stability Assay with Cycloheximide

Cells were cultured with cycloheximide (Sigma-Aldrich) at a concentration of 30 $\mu\text{g}/\text{mL}$ and then incubated for the indicated times in each experiment. Cells were harvested and protein levels were visualized by western blotting with specific antibodies.

Ubiquitination Assay

E14Tg2a or 293T cells were transfected with *pCMV-HA-Oct4* (wild-type or S347A mutant), *pcDNA3-Flag-ubiquitin*, and vectors as indicated, using the jetPEI transfection reagent. At 36 hr after transfection, cells were treated for 4 hr with proteasome inhibitor (MG132, 20 μM) before harvesting and then were disrupted in Nonidet P-40 lysis buffer. Ubiquitination of OCT4 proteins was visualized by immunoprecipitation with a FLAG or HA antibody and western blotting, as indicated.

Statistical Analysis

Values are presented as mean \pm SD. Comparisons among values for all groups were determined using Student's t test. Differences of $p < 0.05$ were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at <https://doi.org/10.1016/j.stemcr.2017.10.017>.

AUTHOR CONTRIBUTIONS

K.B.B. developed the project, performed most of the experiments and data analysis, and wrote the manuscript. D.H.Y. contributed to iPSC generation and cell culture. K.Y.L. designed experiments and contributed to the immunoprecipitation experiments for tandem mass spectrometry analysis and data analysis. K.Y. contributed to iPSC generation and cell culture. J.R. performed the mass spectrometry. D.Y.L. contributed to *in vitro* kinase assay and data analysis. T.A.Z. contributed to *in vitro* kinase assay and data analysis. M.O.K. developed the project and contributed to the gene cloning and data analysis. A.M.B. supervised the project and



reviewed and edited the manuscript. Z.D. directed the project and reviewed the manuscript.

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