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Short Article

Transient Activation of Autophagy via Sox2-Mediated Suppression of mTOR Is an Important Early Step in Reprogramming to Pluripotency

Shuo Wang,[1,2] Pengyan Xia,[1,2] Buqing Ye,[1] Guanling Huang,[1] Jing Liu,[1] and Zusen Fan[1,*]
[1]CAS Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[2]These authors contributed equally to this work
*Correspondence: fanz@moon.ibp.ac.cn
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SUMMARY

Autophagy is an essential cellular mechanism that degrades cytoplasmic proteins and organelles to recycle their components. Here we show that autophagy is required for reprogramming of somatic cells to form induced pluripotent stem cells (iPSCs). Our data indicate that mammalian target of rapamycin (mTOR) is downregulated by Sox2 at an early stage of iPSC generation and that this transient downregulation of mTOR is required for reprogramming to take place. In the absence of Sox2, mTOR remains at a high level and inhibits autophagy. Mechanistically, Sox2 binds to a repressive region on the *mTOR* promoter and recruits the NuRD complex to mediate transcriptional repression. We also detected enhanced autophagy at the four- to eight-cell stage of embryonic development, and a similar Sox2 and mTOR-mediated regulatory pathway seems to operate in this context as well. Thus, our findings reveal Sox2-dependent temporal regulation of autophagy as a key step in cellular reprogramming processes.

INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process that degrades cytoplasmic proteins and organelles to recycle cellular components for cell survival and tissue homeostasis (He and Klionsky, 2009; Kundu and Thompson, 2008). Via double-membraned autophagosomes, long-lived proteins or unwanted organelles are hydrolyzed, and amino acids can be reused for cell renovation (Mizushima and Komatsu, 2011; Mizushima and Levine, 2010). Autophagy provides a recycling system that plays a key role in cellular homeostasis (Mizushima and Komatsu, 2011), tumor suppression (Wang et al., 2012a), antigen processing (Nedjic et al., 2008), antineurodegeneration (Lee et al., 2010), and embryonic development (Mizushima and Levine, 2010). However, it is not yet known whether autophagy is involved in cellular reprogramming.

Somatic cell reprogramming involves epigenetic modification, changes in gene expression, protein degradation, and protein synthesis. Reprogramming resets differentiated cells to a pluripotent state and can be achieved by nuclear transfer, cell fusion, or transduction of certain transcription factors (Yamanaka and Blau, 2010). Originally, four core transcriptional factors (Oct4, Sox2, Klf4 and c-Myc, termed OSKM) were identified as being able to convert differentiated fibroblasts into induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). Subsequently, various methodological changes were made to improve efficiency and reduce carcinogenesis risk during iPSC induction (Yamanaka, 2012). Despite the remarkable progress in reprogramming technology, the underlying molecular mechanisms remain largely elusive. Recently, several reports showed that epigenetic modification starts at an early stage of cell reprogramming, initiating rearrangement of gene expression profiles (Doege et al., 2012; Gu et al., 2011; Koche et al., 2011). Genome-wide analyses and in-depth quantitative proteomics reveal that during the first 3 days of reprogramming, multiple protein profile changes occur in a highly coordinated fashion (Buganim et al., 2012; Golipour et al., 2012; Polo et al., 2012), suggesting that there may be relationships between epigenetic modifications and protein degradation and synthesis. Here we show that autophagy is essential for cell reprogramming. Mechanistically, we show that Sox2 recruits the nucleosome remodeling and deacetylase (NuRD) complex to repress mTOR expression and promote cellular reprogramming through induction of autophagy.

RESULTS

Autophagy Is Required for iPSC Generation

To examine the role of autophagy in somatic cell reprogramming, we generated *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) containing drug-inducible OSKM factors (Carey et al., 2010) (referred to as *4F2A* MEFs hereafter). Atg5 plays a key role in autophagosome formation (Sakoh-Nakatogawa et al., 2013). We found that 3 weeks after initiating culture in embryonic stem cell (ESC) media containing doxycycline, iPSC colonies formed from *Atg5*$^{+/+}$ *4F2A* MEFs, but not from *Atg5*$^{-/-}$ *4F2A* MEFs (Figure 1A), despite comparable expression of the OSKM factors (Figure S1A available online). The iPSCs generated from *Atg5*$^{+/+}$ *4F2A* MEFs expressed pluripotency markers (Figure 1A and Figure S1B) and were able to form teratomas containing cells of all three germ layers (Figure 1B). Atg3 and Atg7 are also required for autophagosome formation (Weidberg et al., 2011). We also found that depletion of Atg3 or Atg7 impaired autophagy and abrogated iPSC formation (Figures S1C and S1D). Restoration of Atg3 or Atg7 into

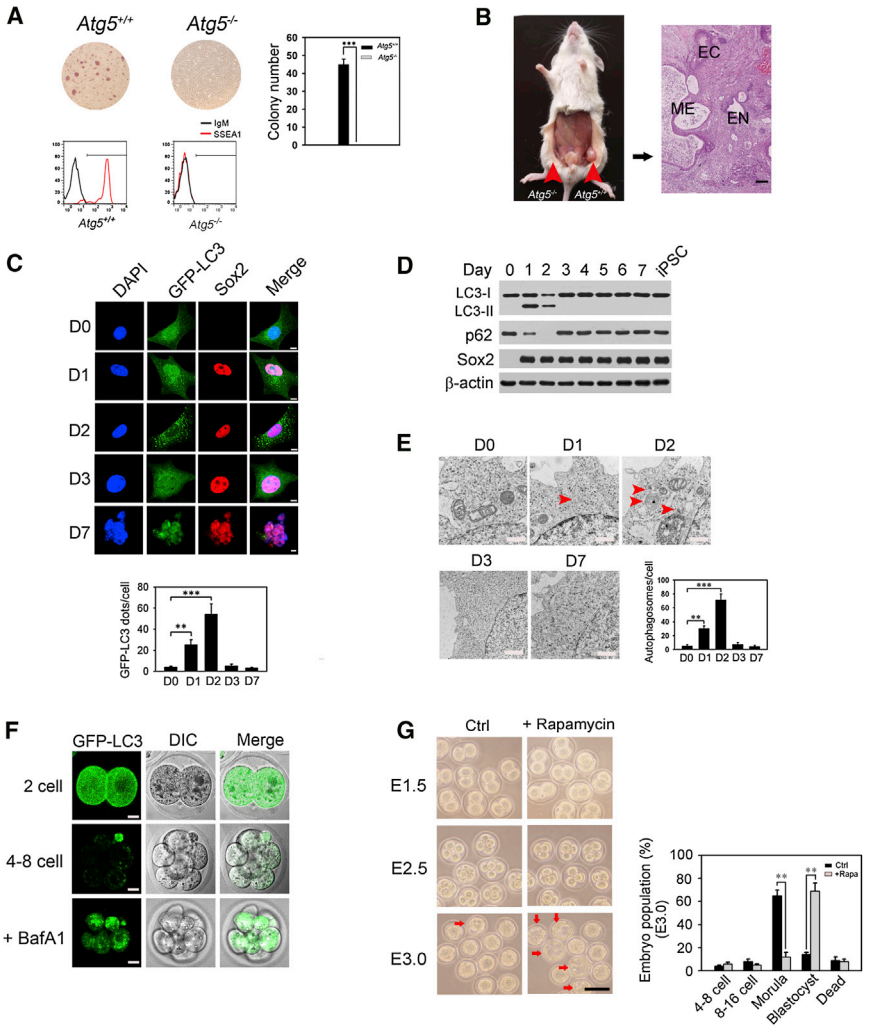

Figure 1. Autophagy Is Essential for iPSC Generation

(A) *Atg5* deficiency impairs iPSC formation. $Atg5^{+/+}$ or $Atg5^{-/-}$ MEFs carrying drug-inducible OSKM factors (hereinafter referred to as *4F2A* MEFs) were cultured in ESC media containing 2 μg/ml doxycycline for 3 weeks and detected for alkaline phosphatase (AP) activity (upper panel) or stained with anti-SSEA-1 antibody (lower panel). Colony numbers per 10^4 cells were calculated and shown as mean ± SD. ***p < 0.001.

(B) iPSCs from $Atg5^{+/+}$, but not $Atg5^{-/-}$; *4F2A* MEFs, can form teratomas. $Atg5^{+/+}$ or $Atg5^{-/-}$; *4F2A* MEFs treated as above were harvested and subcutaneously injected into NOD-SCID mice. One month later, teratomas were collected for H&E staining. EN, endoderm; ME, mesoderm; EC. ectoderm. Scale bar, 200 μm.

(C) Autophagy in iPSC formation. *GFP-LC3*; *4F2A* MEFs were treated with doxycycline for the indicated days and immunostained by anti-Sox2 antibody. Nuclei were visualized by DAPI. GFP-LC3 dots per cell were calculated and shown as mean ± SD. **p < 0.01; ***p < 0.001. Scale bar, 10 μm.

(D) Autophagic flux during iPSC generation. WT *4F2A* MEFs were treated with doxycycline for the indicated days and harvested for immunoblotting.

(E) Autophagosomal structures appear during iPSC generation. WT *4F2A* MEFs were cultured in doxycycline-contained ESC media and autophagosomes were analyzed by electron microscopy and shown as mean ± SD. **p < 0.01; ***p < 0.001. Red arrowhead indicates autophagosome. Scale bar, 500 nm.

(F) Autophagy is enhanced at the four- to eight-cell stage during embryonic development. Embryos at the indicated stages from *GFP-LC3* transgenic mice were isolated, and cultured in OSKM media with or without 20 nM Bafilomycin A1 (BafA1) for 4 hr, and visualized by confocal microscopy. Scale bar, 20 μm.

(G) Rapamycin promotes reprogramming of embryos. Embryos were isolated at embryo day (E) 1.5 and cultured in OSKM media. One microgram per milliliter of rapamycin was added into culture at E2.5 for 4 hr. Distribution of embryonic stage at E3.0 was calculated as mean ± SD (right panel). **p < 0.01. Red arrow indicates blastocyst. Scale bar, 100 μm.

See also Figure S1.

Atg3- or Atg7-silenced cells, respectively, rescued autophagy and restored their capacity to generate iPSCs (Figures S1C and S1D). Overall, these indicate that autophagy is required for iPSC generation. We used *4F2A* MEFs expressing green fluorescent protein (GFP) fused LC3 (GFP-LC3), an autophagosome marker protein, to monitor autophagic activity during reprogramming. Surprisingly, autophagosomes appeared at the first day after OSKM induction and peaked on the following day (Figure 1C). The autophagic flux was verified by conversion of LC3-II and degradation of p62, an autophagic substrate (Mathew et al., 2009) (Figure 1D), and autophagosomal structures were observed by electron microscopy (Figure 1E). Induction of autophagy did not occur in wild-type (WT) MEFs after treatment with doxycycline alone (Figure S1E). In addition, we verified that *Atg5*-deficient cells failed to reduce mitochondrial numbers and reactive oxygen species (ROS) levels after OSKM induction (Figures S1F–S1H), changes that are also viewed as being hallmarks of the establishment of pluripotency (Sena and Chandel, 2012).

A previous study showed that *Atg5*-deficient oocytes could not develop to the four- to eight-cell stage after fertilization with *Atg5* null sperm (Tsukamoto et al., 2008). We also examined embryos at this stage, and found that the level of GFP-LC3 protein reduced rapidly at the four- to eight-cell stage of WT embryos, indicating active autophagy, and was restored by the autophagy inhibitor Bafilomycin A1 (BafA1) (Figure 1F). When we treated embryos with rapamycin to induce autophagy at the four-cell stage, blastocysts appeared as early as embryo day (E) 3.0 (Figure 1G), suggesting that autophagy induction also promotes embryonic development.

Sox2 Initiates Autophagy during Cell Reprogramming

The overall gene expression profile of cells changes substantially during reprogramming (Buganim et al., 2012). To explore which

factors contribute to autophagy induction, we analyzed the mRNA levels of various autophagy-related genes (Atg) at an early stage of iPSC formation. However, all of the Atg genes that we examined showed no significant change after iPSC induction (Figure S2A). We also evaluated the expression levels of genes that are important for autophagy modulation. Interestingly, we observed that *mTOR* mRNA dramatically declined on the first day of OSKM expression (Figure S2B). The mTOR protein level was also repressed for the first two days of the reprogramming process (Figure S2C) but was then restored to basal levels at day 3 of iPSC generation. mTOR exists in two distinct complexes, mTORC1 and mTORC2 (Zoncu et al., 2011). mTORC1 phosphorylates S6K at Thr389 and mTORC2 phosphorylates AKT at Ser473 to facilitate the phosphorylation of AKT at Thr308 (Sarbassov et al., 2005; Zoncu et al., 2011). We found that phosphorylation of both AKT and S6K was completely suppressed during early reprogramming (Figure S2C), indicating that both mTORC1 and mTORC2 are inhibited. Consistent with this observation we also found that polysomes were reduced during the initial days of reprogramming (Figure S2D). Autophagy could be blocked by ectopic expression of WT mTOR but not kinase-dead mTOR (mTOR-KD, an inactive form of mTOR; Brunn et al., 1997) during iPSC induction (Figure S2E). Of note, transient overexpression of WT mTOR blocked iPSC formation, but mTOR-KD had no such activity (Figure S2F). Moreover, the efficiency of iPSC generation could be improved by adding the mTOR inhibitor rapamycin on day 1 of doxycycline induction (Figure S2G). Addition of LY294002, a reversible inhibitor of PI3Ks (Walker et al., 2000) that inhibits autophagy by blocking the activity of Beclin 1-Vps34 complex (Figure S2H), also impaired iPSC generation. The timing of inhibition was also important. When we treated *4F2A* MEFs with rapamycin to block mTOR 3 days after doxycycline induction, no iPSCs were obtained (Figure S2I). These data suggest that an initial short burst of mTOR suppression at an early stage and then restoration of mTOR activity at a later stage are both required for successful reprogramming to take place.

We next analyzed the role of each of the four pluripotency factors in regulation of mTOR expression. Interestingly, ectopic expression of Sox2 dramatically suppressed mTOR expression and promoted the induction of autophagy (Figures 2A–2C), like OSKM expression. However, the other three factors did not impact mTOR expression or autophagy induction (Figures 2A and 2B and Figure S2J). Additionally, we found that Sox2 overexpression could not suppress mTOR expression in HeLa or HEK293T cells (Figure S2K), and we therefore propose that this activity is only manifest during reprogramming. Under physiological conditions, Sox2 is required for the derivation of ESCs, and Sox2 deficiency leads to early embryonic lethality (Avilion et al., 2003; Keramari et al., 2010; Masui et al., 2007). We found that maternal Sox2 protein persisted in the nucleus and cytoplasm of zygotes during embryonic development (Figure 2D), and nuclear Sox2 rose at the four- to eight-cell stage, accompanied with downregulation of mTOR expression (Figures 2D and 2E). Correspondingly, enhanced autophagy occurred at this stage (Figure 1F). We derived $Sox2^{+/+}$; *GFP-LC3* and $Sox2^{-/-}$; *GFP-LC3* embryos through mating of $Sox2^{+/-}$; *EIIa-Cre*; *GFP-LC3* females with $Sox2^{flox/+}$ males. In $Sox2^{-/-}$ embryos, we found that autophagy was impaired (Figure 2F), and the mTOR protein level was not reduced at the four- to eight-cell stage (Figure 2G). These data are consistent with the idea that Sox2 initiates autophagy by suppressing mTOR expression during the cellular reprogramming that occurs in early embryogenesis.

Sox2 Suppresses *mTOR* Expression through Recruitment of NuRD Complex

Sox2 contains a high-mobility-group (HMG) domain, which binds DNA in a sequence-specific manner (Sarkar and Hochedlinger, 2013). We analyzed a 5-kilobase (kb) locus upstream from transcription start sites (TSS) of the *mTOR* gene. Using chromatin immunoprecipitation (ChIP), we found that a −2,200∼−1,200 base pair (bp) region of the *mTOR* promoter upstream from the TSS was occupied by Sox2 protein (Figure 3A). To verify that *mTOR* transcriptional activity is inhibited by Sox2, we used luciferase reporter constructs. Based on these experiments, we identified a −1,800∼−1,600 bp fragment of an *mTOR* promoter as a repressive region where Sox2 bound (Figures 3B and 3C). We then mapped the binding locus on the *mTOR* promoter by electrophoretic mobility shift assay (EMSA) with different oligos from the −1,820∼−1,581 bp fragment, and we determined that the −1,660∼−1,621 bp region of the *mTOR* promoter contains the Sox2 binding locus (Figures 3D and 3E). To investigate a physiological function of this repressive region, we employed transcription activator-like effector nuclease (TALEN) technology to delete the Sox2 binding locus on the *mTOR* promoter (Δ*mTOR*) in MEFs (Figures S3A and S3B). As expected, the binding of Sox2 was abrogated in Δ*mTOR* MEFs (Figure 3F). The expression levels of OSKM in *ΔmTOR*; *4F2A* MEFs were similar to those of WT *4F2A* MEFs (Figure S3C). However, deletion of the repressive locus restored both the mRNA and protein levels of mTOR during early iPSC induction (Figure 3G and Figure S3D) with inhibition of autophagy as a result (Figures 3G and 3H). We treated the *ΔmTOR* cells with rapamycin during the first 2 days of reprogramming and restored the expected level of autophagy (data not shown). Importantly, we did not see production of iPSCs in *ΔmTOR* cells, and this capacity was also restored by rapamycin treatment (Figure 3I). These data suggest that mTOR suppression at an early stage is essential for reprogramming to pluripotency to take place.

To support this conclusion further, we generated an inducible knockdown system for mTOR in *ΔmTOR* MEFs to determine whether silencing of mTOR can rescue reprogramming in these cells. We treated inducible shmTOR *ΔmTOR* MEFs with doxycycline to induce the silencing of mTOR during the first 2 days of reprogramming but also the expression of Oct4, c-Myc, and Klf4 (without Sox2). After 2 days' treatment, Sox2 was also added and mTOR was returned to basal levels by the removal of doxycycline (Figure S3E). Since we used *ΔmTOR* cells, Sox2 could not downregulate mTOR. As expected, iPSCs appeared from shmTOR *ΔmTOR* MEFs (Figure S3F). These data indicate that silencing of mTOR in the absence of Sox2 at an early stage of reprogramming is sufficient for iPSC generation. To examine the role of mTORC1 and mTORC2 in cell reprogramming, we knocked down Raptor and Rictor separately. We found that silencing of Raptor, but not Rictor, in Δ*mTOR* cells at the early stage of reprogramming could rescue iPSC generation in Δ*mTOR* cells (Figure S3G), suggesting that inhibition of mTORC1 is essential for cellular reprogramming. In sum, therefore, Sox2

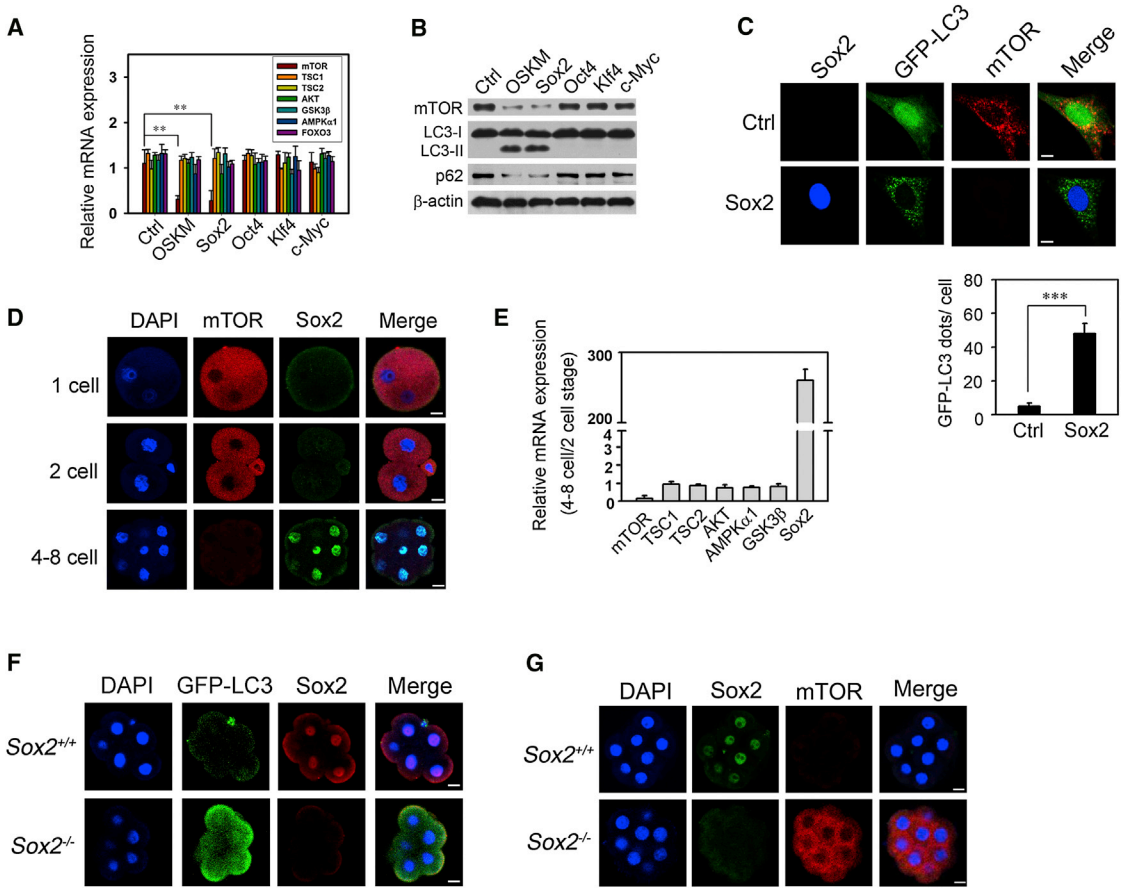

Figure 2. Sox2 Contributes to Autophagy Initiation during iPSC Formation

(A) Sox2 downregulates *mTOR* mRNA during iPSC formation. WT MEFs were infected with retrovirus encoding OSKM factors together or individually and cultured in ESC media for 24 hr. mRNA levels of the indicated genes were analyzed by RT-PCR. Data are representative of three different experiments and shown as mean ± SD. **p < 0.01.

(B) Sox2 contributes to mTOR repression. WT MEFs treated as above were harvested and subjected to immunoblotting with the indicated antibodies.

(C) Sox2 initiates autophagy. *GFP-LC3* MEFs were infected with retrovirus encoding Sox2 or empty vector (Ctrl) and cultured in ESC media for 48 hr, and GFP-LC3 dots were analyzed by confocal microscopy (upper panel) and calculated as mean ± SD (lower panel). ***p < 0.001. Scale bar, 10 μm.

(D) mTOR is downregulated at the four- to eight-cell stage of embryos. Embryos at the indicated stages were isolated and subjected to immunostaining. Nuclei were visualized by DAPI. Scale bar, 20 μm.

(E) Transcription of *mTOR* is impaired at the four- to eight-cell stage. Embryos at the two-cell stage (E1.5) or the four- to eight-cell stage (E2.5) were isolated and harvested for mRNA extraction for RT-PCR. Data are representative of three independent experiments and are shown as mean ± SD.

(F) Autophagy is defective in *Sox2*$^{-/-}$ embryos. Embryos from *Sox2*$^{+/-}$; *EIIa-Cre*; *GFP-LC3* females mated with *Sox2*$^{flox/+}$ males were isolated for immunostaining. Scale bar, 20 μm.

(G) *Sox2* deficiency potentiates mTOR expression. Embryos from *Sox2*$^{+/-}$; *EIIa-Cre* females mated with *Sox2*$^{flox/+}$ males were harvested for immunostaining. Scale bar, 20 μm. We used two different anti-mTOR antibodies (#2972 and #2983, Cell Signaling Technology) for immunoblotting and two different anti-mTOR antibodies (#2983 from Cell Signaling Technology and #sc-8319 from Santa Cruz Biotechnology) for immunofluorescent staining and received similar results. See also Figure S2.

occupies a specific region on the *mTOR* promoter to suppress *mTOR* transcription, leading to initiation of autophagy during iPSC generation.

To explore the mechanism of Sox2-mediated inactivation of *mTOR* transcription, we generated an affinity resin coupled with the −1,660∼−1,621 bp DNA segment and used DNA affinity chromatography to screen for associated proteins that specifically bind to the 40 nucleotides. Notably, several components of the NuRD complex were pulled down from nuclear extracts of MEFs expressing Sox2 (Figure 4A and Figure S4A). The major identified components of NuRD complex (including Mi-2β, LSD1, MTA2, and HDAC1) were confirmed by immunoblotting with their specific antibodies (Figure 4B). Sox2 could also precipitate the major components of NuRD complex in vivo through an immunoprecipitation assay (Figure 4C).

Previous studies have shown that the NuRD complex is a transcriptional repressor that is recruited to gene promoters through interaction with a DNA sequence-specific transcription factor for chromatin remodeling and histone modification (Hu and Wade, 2012). The NuRD complex contributes to deacetylation and demethylation of histones (Wang et al., 2009), which is essential for pluripotency of ESCs (Adamo et al., 2011; Kaji et al., 2006;

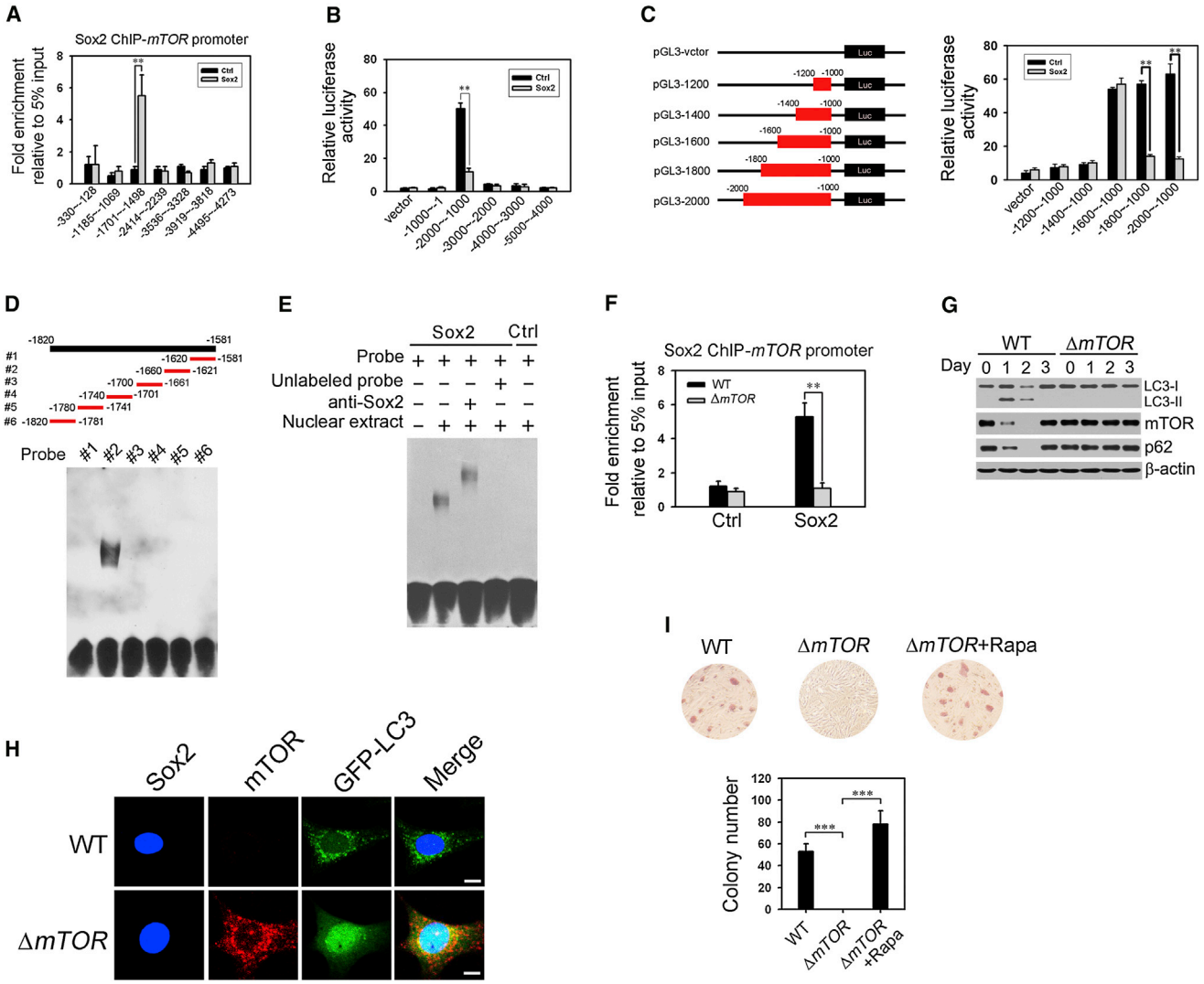

Figure 3. Sox2 Targets *mTOR* Promoter to Repress Its Expression

(A) Sox2 is recruited to the *mTOR* promoter. ChIP analysis monitored binding of Sox2 to *mTOR* promoter in MEFs expressing empty vector (Ctrl) or Sox2. Data represent at least three separate experiments and are shown as mean ± SD. **$p < 0.01$.

(B and C) Sox2 suppresses *mTOR* transcription. Different loci of the *mTOR* promoter were constructed into pGL3 vector, transfected into MEFs expressing empty vector (Ctrl) or Sox2, and subjected to luciferase reporter assay. Data are representative of three different experiments and are shown as mean ± SD. **$p < 0.01$.

(D) Sox2 binds to the *mTOR* promoter. Nuclear extracts from MEFs expressing Sox2 were monitored by EMSA assay with different probes as shown in the upper panel.

(E) Sox2 binds a specific locus on the *mTOR* promoter. EMSA assay monitored Sox2 binding of *mTOR* promoter with the specific 40 nucleotide probe (#2 probe as in 3D) with nuclear extracts from MEFs expressing empty vector (Ctrl) or Sox2. Anti-Sox2 antibody was added for supershift assay, and unlabeled probes were added for competitive reaction.

(F) The repressive locus deletion (*ΔmTOR*) abolishes Sox2 binding to *mTOR* promoter. WT or *ΔmTOR* MEFs expressing empty vector (Ctrl) or Sox2 were subjected to ChIP assay by our use of anti-Sox2 antibody. Data are representative of three independent experiments and are shown as mean ± SD. **$p < 0.01$.

(G) *ΔmTOR* abrogates mTOR downregulation and autophagy. WT or *ΔmTOR*; *4F2A* MEFs were cultured in doxycycline-contained ESC media for the indicated days and harvested for immunoblotting.

(H) mTOR expression and autophagy induction are impaired in *ΔmTOR* MEFs. WT or Δ*mTOR*; *GFP-LC3*; *4F2A* MEFs were cultured in doxycycline-contained ESC media for 48 hr and subjected to immunostaining. Scale bar, 10 μm.

(I) iPSC generation is blocked in *ΔmTOR* MEFs. *4F2A* WT or *4F2A*; *ΔmTOR* MEFs were treated with or without 1 μg/ml rapamycin (Rapa) for 48 hr and cultured in ESC media containing doxycycline for 3 weeks. The iPSCs were visualized by AP staining. Data are representative of three different experiments and are shown as mean ± SD. ***$p < 0.001$. Similar results were obtained by using two different anti-mTOR antibodies for immunoblotting and immunofluorescence assays as described above.

See also Figure S3.

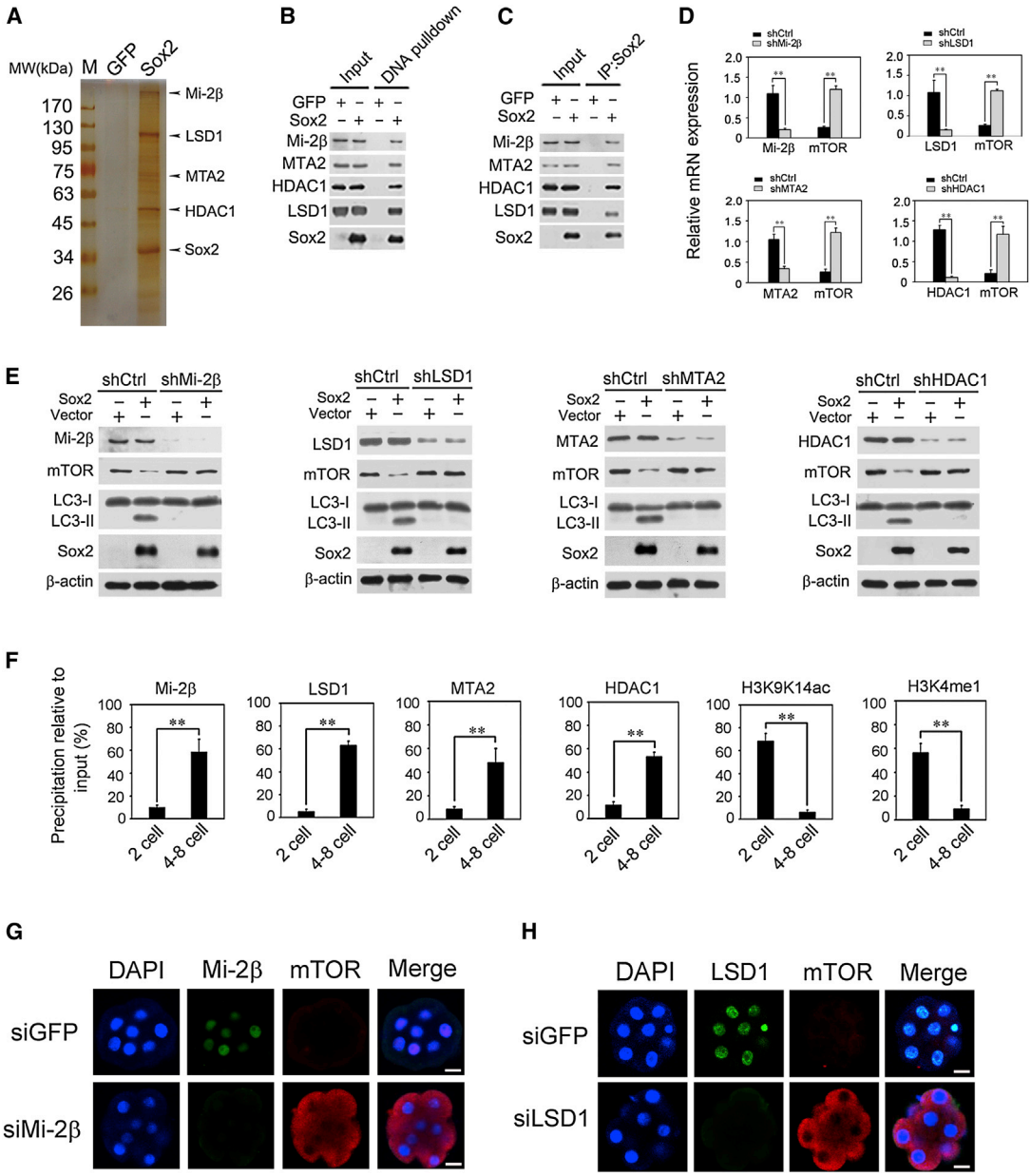

Figure 4. Sox2 Recruits the NuRD Complex to Inhibit *mTOR* Transcription

(A) Identification of NuRD complex by a DNA pulldown assay. The binding region (40 nt) on the *mTOR* promoter of Sox2 was conjugated to CNBr activated beads. Nuclear extracts from MEFs expressing GFP or Sox2 were incubated with DNA-conjugated beads for 20 min and the precipitates were subjected to SDS-PAGE for silver staining and mass spectrometry. M, molecular weight (MW) marker.

(B) The identified components of the NuRD complex were confirmed by western blotting.

(C) Sox2 can precipitate the NuRD complex in vivo. MEFs expressing GFP or Sox2 were harvested for immunoprecipitation with anti-Sox2 antibody. Immunoprecipitates were detected by immunoblotting with the indicated antibodies.

(D) Silencing of NuRD complex components restores *mTOR* mRNA levels. MEFs were infected with retrovirus encoding shRNA against scramble sequence (shCtrl) or components of NuRD complex, as well as Sox2 or empty vector, and cultured in ESC media for 24 hr followed by RT-PCR analysis. Data are representative of at least three experiments and are shown as mean ± SD. $^{**}p < 0.01$.

(E) The NuRD complex is required for autophagy flux induced by Sox2. MEFs were infected with retrovirus encoding the indicated shRNA and Sox2 or empty vector, cultured in ESC media for 24 hr, and harvested for immunoblotting. β-actin was used as a loading control.

(F) The NuRD complex is recruited to *mTOR* promoter during early embryonic development. Embryos from the two-cell stage or the four- to eight-cell stage were determined by ChIP analysis. Data represent three separate experiments and are shown as mean ± SD. $^{**}p < 0.01$.

(G and H) Knockdown of NuRD complex components impairs mTOR downregulation at the four- to eight-cell stage of embryos. Mi-2β (G) or LSD1 (H) was silenced with siRNA duplexes at E1.5. Embryos were cultured in OSKM media for 24 hr and subjected to immunostaining. Scale bar, 20 μm. Similar results were obtained by using two different anti-mTOR antibodies for immunoblotting and immunofluorescence assays as described above.

See also Figure S4.

Zhu et al., 2009). However, its functions in somatic cell programming are unclear. Using ChIP assays, we found that Sox2 could recruit the NuRD complex to the *mTOR* promoter (Figure S4B). Consistently, the NuRD complex suppressed the addition of H3K9K14ac and H3K4me1 transcriptional activation marks on the *mTOR* promoter (Figure S4B). We knocked down components of the NuRD complex individually and found that depletion of each NuRD component resulted in restoration of *mTOR* mRNA (Figure 4D). Intriguingly, knockdown of NuRD complex components also restored the mTOR protein level and abrogated Sox2-mediated autophagy induction (Figure 4E and Figure S4C). We then looked at the binding of the NuRD complex to the *mTOR* promoter at 3 days after the initiation of iPSC induction, and we found that the NuRD complex disassociated from the *mTOR* promoter at this time (Figure S4D) and that H3K4me1 and H3K9K14ac returned to basal levels at that later stage. These data suggest that dynamic binding of NuRD complex to the mTOR promoter correlates with the restoration of mTOR during the reprogramming process. Furthermore, disruption of the NuRD complex impaired iPSC formation (Figure S4E). We also silenced mTOR during the initial reprogramming period in NuRD-complex-silenced cells and found that reprogramming was again rescued (Figure S4F). Together, these results suggest that NuRD-complex-mediated suppression of *mTOR* transcription contributes to somatic cell reprogramming.

We then looked at binding of the NuRD complex to the *mTOR* promoter during embryonic development. We found that the NuRD complex was recruited to the *mTOR* promoter at the four- to eight-cell stage (Figure 4F), and H3K9K14ac and H3K4me1 were substantially reduced at the *mTOR* promoter. Notably, silencing of NuRD complex components in embryos at the four- to eight-cell stage maintained the mTOR protein level (Figures 4G and 4H and Figure S4G). These data indicate that NuRD-complex-mediated suppression of mTOR is also involved in early embryonic development and is likely to be the relevant mechanism for autophagy induction in this context as well.

DISCUSSION

In this study, we outline a pathway in which Sox2-mediated suppression of *mTOR* expression leads to a transient increase in autophagy early in the reprogramming process that is required for successful reprogramming to take place. mTOR is a key modulator of aging, protein synthesis, glycolysis, and autophagy (Johnson et al., 2013). Previous studies on the role of mTOR in reprogramming have led to some apparently contradictory findings. For example, He et al. found that Tsc2 deficiency and high concentrations of rapamycin both blocked generation of iPSCs (He et al., 2012). Our study provides a molecular explanation for this observation by showing that an initial short burst of mTOR suppression at an early stage and mTOR activity restoration at a later stage are both required for reprogramming to be completed successfully. Our findings also reveal that the dynamic repression and restoration of mTOR activity are precisely regulated by the Sox2-NuRD complex during the reprogramming process. Further studies are needed to elucidate the underlying mechanism that mediates termination of mTOR suppression and autophagy.

Some of our results regarding mTOR expression during early embryogenesis differ from those reported previously. González et al. (2012) showed that mTOR protein levels were consistent from oocyte to blastocyst. However, these two studies used different mouse strains. We used C57BL/6 mice, while González et al. used ICR mice. It is possible that strain-specific differences in mTOR epitope detection or early embryogenesis pathways could contribute to the difference in results, particularly because the efficiency of ESC derivation is known to differ between mouse strains (Hanna et al., 2010).

Sox2 is a core factor for cell reprogramming. We found that Sox2 occupies a −1,660∼−1,621 bp segment upstream of the TSS of the *mTOR* gene during the early stages of reprogramming. Our data indicate that Sox2 recruits the NuRD complex that downregulates H3K9K14ac and H3K4me1, leading to transcriptional inhibition of *mTOR*. Silencing of NuRD complex components impaired the repression of *mTOR* expression and induction of autophagy. After initiation of cellular reprogramming, the NuRD complex disassociates from the *mTOR* promoter to restore mTOR expression for the rest of the reprogramming process. Our findings therefore reveal that the NuRD complex participates in the regulation of somatic cell reprogramming. In addition, the NuRD complex is essential for embryonic development and deficiency of the NuRD complex causes early embryonic lethality (Hu and Wade, 2012). During embryonic development, we found that the NuRD complex downregulated H3K9K14ac and H3K4me1 of the *mTOR* promoter, and silencing of NuRD components restored the mTOR protein level, suggesting that the NuRD complex epigenetically regulates the *mTOR* promoter in a manner that is essential for embryonic cellular reprogramming. Further understanding of the mechanisms of somatic reprogramming will help us not only to promote efficiency of iPSC generation but also to shed light on the developmental biology of early embryogenesis.

EXPERIMENTAL PROCEDURES

Generation of iPSCs

MEFs were obtained at E14.5 of *4F2A* embryos, *GFP-LC3*; *4F2A* embryos, or *Atg5*$^{-/-}$; *4F2A* embryos and supplied with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 15% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. After one passage, MEFs were plated on a 6-well dish and cultured in ESC media containing 2 μg/ml doxycycline. After 7 days, MEFs were plated on mitomycinC-treated B6 MEF feeders. After 3 weeks in ESC media containing doxycycline, iPSC colonies were analyzed by an alkaline phosphatase detection kit (Millipore).

Generation of *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs

Atg5$^{flox/flox}$ mice were crossed with *Ella-Cre* mice to obtain *Atg5*$^{+/-}$ mice. *Atg5*$^{+/-}$ mice were crossed with *4F2A* transgenic mice or *GFP-LC3* transgenic mice to obtain *4F2A*; *Atg5*$^{+/-}$ and *GFP-LC3*; *Atg5*$^{+/-}$ mice. Next, *4F2A*; *Atg5*$^{+/-}$ mice were crossed with *GFP-LC3*; *Atg5*$^{+/-}$ mice. The pregnant mice were sacrificed and E14.5 embryos were isolated and digested into single MEFs. The genotypes of MEFs (*4F2A*; *GFP-LC3*; *Atg5*$^{+/+}$ MEFs and *4F2A*; *GFP-LC3*; *Atg5*$^{-/-}$ MEFs) were analyzed by PCR.

Immunofluorescence Assay

Immunofluorescence assays were performed as previously described (Wang et al., 2012b; Xia et al., 2013). Briefly, MEFs were grown on 0.01% poly-L-Lysine-treated coverslips and infected with retrovirus encoding the indicated proteins for 24 hr. Next, cells were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 20 min at room temperature (RT) followed by permeabilization with 0.5% Triton X-100 for 20 min at RT. Ten percent donkey serum was

used for blocking followed by incubation of primary antibodies for 2 hr at RT. After being washed three times with PBS, the coverslips were stained with Alexa 488-, Alexa 594-, or Alexa 405-conjugated secondary antibodies. For embryonic assay, embryos were collected from pregnant mice at the indicated times, treated with acidic tyrode for 30 s to remove zona pellucid, fixed with 4% PFA for 20 min at RT, and permeabilized with 0.5% Triton X-100 in PBS for 20 min at RT. After being blocked with 10% donkey serum, embryos were incubated with primary antibodies at 4°C overnight and then with secondary antibodies for 1 hr at RT. Images were visualized by laser scanning confocal microscopy (Olympus FV500).

Statistical Analysis

Student's t test was used as statistical analysis through our use of Sigma Plot. For other methods see the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.10.005.

AUTHOR CONTRIBUTIONS

S.W. designed and performed experiments, analyzed data, and wrote the paper, and P.X. performed experiments and analyzed data. B.Y. analyzed data; G.H. and J. L. constructed plasmids; and Z.F. initiated the study and organized, designed, and wrote the paper.

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