USP26 functions as a negative regulator of cellular reprogramming by stabilising PRC1 complex components

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Despite much progress in the comprehension of the complex process of somatic cell reprogramming, many questions regarding the molecular mechanism of regulation remain to be answered. At present, the knowledge on the negative regulation of reprogramming process is indeed poor in contrary to the identification of positive regulators. Here we report for the first time that ubiquitin-specific protease 26 negatively regulates somatic cell-reprogramming process by stabilizing chromobox (CBX)-containing proteins CBX4 and CBX6 of polycomb-repressive complex 1 through the removal of K48-linked polyubiquitination. Thus, accumulated CBX4 and CBX6 repress the expression of pluripotency genes, such as Sox2 and Nanog, through PRC1 complexes to ubiquitinate histone H2A at their promoters. In all, our findings have revealed an essential role for ubiquitin-specific protease 26 in cellular reprogramming through polycomb-repressive complex 1.
Somatic cells can be reprogrammed by the transduction of four key transcription factors (Oct4, Sox2, Klf4, and cMyc) to give rise to induced pluripotent stem cells (iPSCs)\(^1\). With a view to utilizing iPSCs for the studies involving regenerative medicine and drug screening, many researchers have focused on the dissection of the molecular mechanisms of cellular reprogramming\(^2\). However, this reprogramming process is inefficient and variable. Therefore, understanding other factors particularly negative regulators of cellular reprogramming should be instrumental for developing a more reliable and accelerated process for obtaining iPSCs. In the last few years, many epigenetic factors have been identified to play critical roles and reprogramming somatic cells into a pluripotent state\(^3\)–\(^7\). For instance, histone methyltransferase and demethylase, such as Wdr5, SUV39H1/2, Setdb1, Utx, Jmjd3, and Dot1L, either positively or negatively regulate the kinetics and efficiency of cellular reprogramming\(^8\)–\(^10\). Despite theoretical role of PRC1 in the repression of specific genes in differentiation\(^11\), the precise mechanisms that control the dynamic of the various protein subunits of the PRC1 complexes during cellular reprogramming are poorly understood.

The core proteins of PRC1 complex comprises RING1A or RING1B with one of six polycomb group RING finger (PCGF) proteins, which can bind to RING1A or RING1B within the E3 catalytic unit of the PRC1 complex\(^12\). On the basis of the composition of various protein subunits, these PRC1 complexes are classified as PRC1.1–PRC1.6 families\(^13\). PRC1 complexes can ubiquitinate histone 2A lysine 119 (H2AK119), repressing cell lineage-specific or pluripotency gene transcription\(^14\),\(^15\). The canonical PRC1 variants (PRC1.2 and PRC1.4) contain PCGF2 and PCGF4, respectively, RING1A/B, polyhomeotic (PHC) and chromobox-containing (CBX) proteins, such as CBX2, CBX4, CBX6, or CBX7, and can specifically recognize H3K27me3 on H3 histone\(^12\). The dynamic interchange of PRC1.2 and PRC1.4 subunits modulates the balance between self-renewal and lineage commitment in embryonic stem cells (ESCs)\(^16\).

In this study, we report that the post-translational regulation of PRC1 components CBX4 and CBX6 by ubiquitination is critical for reprogramming. Importantly, our systematic investigation demonstrates that deubiquitinase ubiquitin-specific protease 26 (USP26) acts as a potent negative regulator in the process of somatic cell reprogramming into iPSCs by stabilizing CBX4 and CBX6 through the USP26-mediated removal of K48-linked ubiquitination. We further show that the ectopic expression of USP26 blocks reprogramming by repression of pluripotency genes, such as Sox2 and Nanog, mediated through CBX4 and CBX6 accumulation. By contrast, knockdown of USP26 enhances the efficiency of reprogramming by reactivating Sox2 and Nanog through degradation of CBX4 and CBX6. To our knowledge, this is the first demonstration of the coordination of the USP26-CBX4/CBX6 axis in the negative regulation of somatic cell reprogramming, thus providing new insights into molecular mechanisms by which USP26 regulates the specific components of PRC1 complexes.

**Results**

Functional role of USP26 in cellular reprogramming. Recent studies show that the ubiquitin (Ub)–proteasome system regulates stem cell pluripotency and cellular reprogramming by ubiquitination-mediated degradation of key pluripotency factors\(^17\), but the function and mechanisms of many USP proteins in cellular reprogramming remain poorly understood. To define the potential functions of the USP family proteins in cellular reprogramming, we screened USP family members, using short hairpin RNA (shRNA) knockdown, based on their ability to increase or inhibit the reprogramming efficiency of mouse embryonic fibroblast (MEF) into iPSCs. For this purpose, shRNAs for 48 mouse USP family members or reference shRNA.
LIF withdrawal and treatment with 1 μM Dox inducible GFP-tagged Usp26 or empty vector lentivirus. After ESC colonies formed, then low-dose Dox was added for 1 day pre-selection. Day 0 was defined as the day when pre-selected GFP-positive colonies were cultured in iSF1 medium with high-dose Dox (2 μg/ml), individual colonies were tracked and taken pictures on Usp26-expressing days 1, 2, 3, and 4. β-actin was used as a loading control.

Experimental scheme of Usp26-induced ESC differentiation. ES cells were transduced with Dox-inducible GFP-tagged Usp26 or empty vector lentivirus. After ESC colonies formed, then low-dose Dox (0.1 μg/ml) was added for 1 day pre-selection. Day 0 was defined as the day when pre-selected GFP-positive colonies were cultured in iSF1 medium with high-dose Dox (2 μg/ml), individual colonies were tracked and taken pictures on Usp26-expressing days 1, 2, 3, and 4. β-actin was used as a loading control.

**Fig. 2** USP26 is required for ESC differentiation. **a** Real-time qPCR analysis of mouse Usp26 mRNA expression in MEFs, iPSCs, and ESCs, **p < 0.01 compared to MEFs.** **b** Western blot analysis of USP26 protein expression in MEFs, iPSCs, and ESCs. **Real-time qPCR analysis of mouse Usp26 and Nanog mRNA expression during Dox-induced OSKM-mediated MEF reprogramming on days 0, 4, 8, and 12, **p < 0.05 compared to Usp26, **p < 0.05 compared to day 0. **d** Real-time qPCR analysis of mouse Usp26 and Nanog mRNA expression in ESCs after LIF withdrawal and treatment with 1 μM RA on days 1, 2, 3, and 4, **p < 0.05 compared to Usp26, **p < 0.05 compared to day 1. **e** Western blot analysis of USP26, OCT4, and NANOG protein expression in ESCs after LIF withdrawal and treatment with 1 μM RA at days 0, 2, 4, and 6. β-actin was used as a loading control. **f** Experimental scheme of Usp26-induced ESC differentiation. ES cells were transduced with Dox-inducible GFP-tagged Usp26 or empty vector lentivirus. After ESC colonies formed, then low-dose Dox (0.1 μg/ml) was added for 1 day pre-selection. Day 0 was defined as the day when pre-selected GFP-positive colonies were cultured in iSF1 medium with high-dose Dox (2 μg/ml), individual colonies were tracked and taken pictures on Usp26-expressing days 1, 2, 3, and 4. β-actin was used as a loading control. **g** Bright field (BF) and fluorescent microscopic images of ESC morphology with Dox-inducible GFP-tagged mUsp26 overexpression or with GFP-tagged empty vector (EV). **h** Bright field images of ESC morphology of wild-type (WT) or Usp26 knockout (KO) ESCs treated with RA. WT or Usp26 KO ESCs were cultured in ES differentiation medium (LIF withdrawal with 1 μM RA). Individual colonies were tracked and photographed on days 0, 2, 4, and 6 under microscope. The data are presented as means ± SD from three independent experiments. **a, c, d** Two-way ANOVA for multiple comparisons.

from Dharmaco mouse shRNA library (with a GFP expression cassette in the vector) were selected based on their ability to knockdown their corresponding genes. We used at least two individual shRNAs of each gene with high knockdown efficiency (>70%) for screening. Dox-inducible Oct4-Sox2-Klf4-cMyc (OSKM) transgenic MEFs were transduced with individual lentiviral shRNAs specific for USP gene. The reprogramming efficiency was determined by alkaline phosphatase (AP) staining after doxycycline (Dox) induction for 12 days (Fig. 1a). AP staining showed that knockdown of Usp26 in MEFs (Supplementary Fig. 1a) generated ~400 iPSC colonies, which was approximately fourfold higher than MEFs transduced with control shRNA (Fig. 1b, c). Conversely, iPSC colony numbers decreased upon Usp20 knockdown (Fig. 1c). The knockdown of other USP family member proteins, such as USP30 and USP52, was also found to exert effects on reprogramming efficiency.
albeit at much lesser extent (less than twofold). In contrast, the overexpression of Usp26 decreased the number of AP+ colonies (Fig. 1d, e), while overexpression of Usp20 increased the number of AP+ colonies (Fig. 1d). The previous study has shown that Usp20 increases the expression of HIF1A, which is reported as a metabolic switch for an early stage of iPSC20. Usp26 shRNA transduction efficiency was characterized by shRNA GFP expression in iPSC colonies (Supplementary Fig. 1d) and by quantitative polymerase chain reaction (qPCR) of Usp26 (Supplementary Fig. 1a). The reprogramming efficiency of Usp26 was also evaluated by NANOG immunofluorescent staining (Supplementary Fig. 1b, c). The role of Usp26 in somatic cell reprogramming was further confirmed by the development of teratomas, containing several tissues, such as adipose tissue, neural rosette-like tissue, and gut-like epithelial tissue (Supplementary Fig. 1e) and by protein immunostaining of pluripotency markers, such as OCT4, NANOG, and DPPA4 (Fig. 1f). Cumulatively, our data suggest that Usp26 plays an important role in cellular reprogramming into a pluripotent state.

### USP26 expression promotes ESC differentiation.

To study the function of USP26 in pluripotency, we compared mRNA and protein levels of USP26 in MEFs, ESCs, and iPSCs. USP26 mRNA and protein levels in MEFs were higher than those in iPSCs or ESCs (Fig. 2a, b). As expected, Nanog, a known pluripotency gene, increased dramatically during Dox-induced OSKM MEF reprogramming and decreased upon removal of leukemia inhibitory factor (LIF) during retinoic acid (RA)-induced ESC differentiation (Fig. 2c, d). However, USP26 mRNA gradually decreased during Dox-induced OSKM-mediated MEF reprogramming and increased during ESC differentiation (Fig. 2c, d).

Similarly, USP26 protein levels increased from day 0 to day 6 upon addition of RA (Fig. 2e). Furthermore, infection of ESCs with GFP-tagged mouse (m) USP26 led to ESC differentiation in an RA-independent manner (Fig. 2f). To further understand the role of USP26 in ES cells, we generated Usp26 knockout ESCs using a CRISPR/Cas9 technology. Since the expression level of USP26 is low in ESCs, we tested the knockout efficiency of USP26 using MEFs (Supplementary Fig. 2c, d). Wild-type (WT) ESCs began differentiating within 2 days after RA stimulation, whereas Usp26 knockout ESCs maintained ESC morphology even after incubation with RA for 6 days (Fig. 2g) and began differentiating on day 8 (Supplementary Fig. 2a). However, protein levels of pluripotency and Polycomb markers, such as NANOG and CBX7, in Usp26 knockout cells, remain similar during a 6-day RA induction of ESCs (Supplementary Fig. 2b). These results suggest that USP26 expression promotes ESC differentiation.

### USP26 inhibits expression of pluripotency core genes.

To study the effects of Usp26 knockdown on gene expression during MEF reprogramming, we performed qPCR and measured the expression levels of 90 genes, which have been reported to be involved in reprogramming (Supplementary Data 1). Our results showed that after Usp26 knockdown during Dox-induced OSKM-mediated MEF reprogramming led to increased mRNA levels of Sox2 and Nanog, but not Oct4, compared with control (Fig. 3a). Overexpression of Usp26 inhibited Sox2 and Nanog gene expression during Dox-induced OSKM-mediated MEF reprogramming (Fig. 3b). These data suggest that the expression of Sox2 and Nanog gene is regulated by Usp26 during Dox-induced OSKM-mediated MEF reprogramming. Overexpression of Usp26 in ESCs led to decreased mRNA levels of Sox2 and Nanog gene expression during Dox-induced OSKM-mediated MEF reprogramming (Fig. 3c).
Supplementary Fig. 4d), protein levels of CBX4 and CBX6 were also observed in differentiated ESCs on day 6 revealed binding of USP26, CBX4, CBX6, H2A-ub1, and H3K27me3 to the Sox2, Nanog, and Cbx7 promoters (Supplementary Fig. 5a). This suggests that the PRC1 components are potential targets of USP26. Accumulated CBX4 and CBX6 inhibit pluripotent genes. Next, we sought to determine whether increased CBX4 and CBX6 levels affect pluripotent genes. ChIP-qPCR of the promoters of Sox2, Nanog, Oct4, and Cbx7 in USP26-differentiated ESCs on day 6 revealed binding of USP26, CBX4, CBX6, H2A-ub1, and H3K27me3 to the Sox2, Nanog, and Cbx7 promoters (Supplementary Fig. 5a). This suggests that USP26 and CBX4 or CBX6, but not CBX7, were only observed in differentiating ESCs (Fig. 4c). To determine whether USP26 interacts with the PRC1 components RING1A, CBX4, and CBX6 independent of each other, we generated RING1A, CBX4, or CBX6 knockout 293T cells using CRISPR/Cas9 technology (Supplementary Fig. 3c, d). IP experiments showed binding of FLAG-USP26 and CBX4 or CBX6 in RING1A KO 293T cells (Fig. 4c), suggesting that USP26 and CBX4 or CBX6 interactions are RING1A-independent. However, in CBX4 or CBX6 knockout 293T cells, USP26 did not interact with RING1A, suggesting that USP26 and RING1A interactions are dependent on the presence of CBX4 or CBX6 (Fig. 4c). These protein–protein interactions in RING1A KO, CBX4 KO, or CBX6 KO 293T cells were further demonstrated using HA-tagged PRC1 proteins and FLAG-tagged USP26 (Supplementary Fig. 3b, e, f). Taken together, these results suggest that USP26 physically binds to components of the PRC1 complex, including RING1A, CBX4, and CBX6, and the interaction between USP26 and CBX4 or CBX6 is independent of the presence of RING1A.

USP26 stabilizes CBX4 and CBX6. To further identify molecular mechanisms of how USP26 regulates CBX4 and CBX6, we measured Cbx4 and Cbx6 mRNA and protein levels in MEFs with or without USp26 overexpression. Although USp26 ectopic expression in MEFs did not change Cbx4 or Cbx6 mRNA levels (Fig. 5a and Supplementary Fig. 4a), we found that USp26 expression resulted in increased CBX4 and CBX6 protein levels (Fig. 5b and Supplementary Fig. 4b), thus suggesting that USp26 regulates CBX4 and CBX6 in a post-transcriptional manner. Furthermore, with increasing expression of Dox-inducible USP26 in 293T cells (Supplementary Fig. 4c) and in ES cells (Fig. 5c and Supplementary Fig. 4d), protein levels of CBX4 and CBX6 were also increased. Additionally, CBX4 and CBX6 protein levels were also significantly increased upon addition of the proteasome inhibitors, MG132 or Lactacystin in 293T cells (Supplementary Fig. 4e). In MEF cells, which express USP26 endogenously, we also found that proteasome inhibitors have less effect on CBX4 and CBX6 accumulation (Supplementary Fig. 4f), USP26 stabilizes CBX4 and CBX6 by reducing proteasomal degradation. Protein modifications by K48-linked poly-Ub chains are well-established signals for recognition and initiation of degradation by the 26S proteasome complexes. Therefore, we analyzed K6-linked, K11-linked, K27-linked, K29-linked, K33-linked, K48-linked, and K63-linked polyubiquitination of CBX4 and CBX6 by IP (Supplementary Fig. 4g). We found that both CBX4 and CBX6 were modified by K48-linked polyubiquitination, which could be removed by USP26 (Fig. 5d). We also generated a catalytically inactive USP26 mutant by replacing the active-site cysteine with a serine residue (C304S), and a deletion mutant by deleting the conserved domain (Δ295-312). Neither of these USP26 mutants could remove K48-linked polyubiquitination of CBX4 or CBX6 (Fig. 5e), indicating that the deubiquitase activity of USP26 is required for the regulation of CBX4 or CBX6 protein stability.

USP26 binds to CBX4 and CBX6 in RING1A-independent manner. To determine whether USP26 targets subunits of the PRC1 complexes, we performed immunoprecipitation (IP) experiments to screen for protein–protein interactions. Hemagglutinin (HA)-tagged PRC1 components, including RING1A, RING1B, RYBP, PCGF1, PCGF2, BM1, CBX4, CBX6, CBX7, and CBX2, were transfected into 293T cells along with FLAG-tagged human (h) USP26. Components of the PRC1.2 complex including RING1A, PCGF2, CBX4, CBX6, and CBX7 interacted with USP26, but components of other PRC1 families, such as RING1B (all PRC1s), RYBP (all PRC1s), PCGF1 (PRC1.1), BM1 (PRC1.4; Fig. 4a) as well as KDM2B (PRC1.1) and PHC1 (PRC1.4; Supplementary Fig. 3a), did not interact with USP26. Components of the PRC2 complex EZH1, EED, and EZH2 were also tested, but no interactions were observed (Supplementary Fig. 3a). Since the undifferentiated ESCs expressed CBX7 but no USP26, endogenous interactions between USP26 and CBX4 or CBX6, but not CBX7, were only observed in differentiating ESCs (Fig. 4b). To determine whether USP26 interacts with the PRC1 components RING1A, CBX4, and CBX6 independent of each other, we generated RING1A, CBX4, or CBX6 knockout 293T cells using CRISPR/Cas9 technology (Supplementary Fig. 3c, d). IP experiments showed binding of FLAG-USP26 and CBX4 or CBX6 in RING1A KO 293T cells (Fig. 4c), suggesting that USP26 and CBX4 or CBX6 interactions are RING1A-dependent. However, in CBX4 or CBX6 knockout 293T cells, USP26 did not interact with RING1A, suggesting that USP26 and RING1A interactions are dependent on the presence of CBX4 or CBX6 (Fig. 4c). These protein–protein interactions in RING1A KO, CBX4 KO, or CBX6 KO 293T cells were further demonstrated using HA-tagged PRC1 proteins and FLAG-tagged USP26 (Supplementary Fig. 3b, e, f). Taken together, these results suggest that USP26 physically binds to components of the PRC1 complex, including RING1A, CBX4, and CBX6, and the interaction between USP26 and CBX4 or CBX6 is independent of the presence of RING1A.

NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-00301-4 | www.nature.com/naturecommunications

Sox2, Nanog, and Oct4 compared with control cells (Fig. 3c). This inhibition of pluripotent gene expression began as early as 2 days after induction of ESC differentiation without RA. These results suggest that USP26 regulates the expression of pluripotency genes during cellular reprogramming to a pluripotent state and ESC differentiation. We also found significant decrease in the gene expression of Cbx7, a Polycomb protein, upon Usp26-induced ESC differentiation (Fig. 3c). Upon Dox-induced OSKM-mediated MEF reprogramming, Cbx7 gene expression increased when Usp26 was knocked down (Fig. 3a) and decreased when Usp26 was overexpressed (Fig. 3b). In addition, based on qPCR analysis (Supplementary Table 1), Cbx7, Nanog, and Sox2 were identified as the most significantly changed genes in MEFs during reprogramming with Usp26 knockdown. Overall, these data suggest that the PRC1 components are potential targets of USP26.
expression by Usp26. These results suggest that Cbx4 or Cbx6 alone is sufficient for downregulating Sox2, Nanog, or Cbx7 expression. To understand how Usp26 expression is regulated, we assessed the promoter-binding activity of Usp26 by PRC1 subunits using the ChIP-qPCR assay during RA-induced ESC differentiation, and observed that PRC1 components PCGF2 and Cbx7 occupation as well as H2A-ub1 modifications decreased significantly in Usp26 promoter (Supplementary Fig. 5d), thus indicating that CBX7 containing PRC1 complex is a potential repressor of Usp26. To study whether Usp26 can regulate CBX7 directly or through interactions with CBX4 and CBX6, we performed luciferase assay and found that overexpression of Usp26 alone could not inhibit promoter activities of these pluripotency genes. However, when Usp26 was expressed with either Cbx4 or Cbx6, significantly decreased promoter activities of Sox2, Nanog, and Cbx7 were observed (Supplementary Fig. 5g), suggesting that Usp26 interacts with Cbx4 or Cbx6 alone or both in Cbx7 promoter for CBX7 suppression. Taken together, these results provide molecular mechanisms of Usp26-mediated increased CBX4 and CBX6 protein stability for suppressing pluripotent gene expression.

**Ectopic expression of CBX4 and CBX6 blocks reprogramming.**
To determine how Polycomb proteins affect MEF reprogramming, we used shRNA to knockdown Cbx4, Cbx6, Cbx7, and Ring1a in MEFs. After 12 days of Dox treatment, knockdown of Cbx4 or Cbx6, but not Cbx7 or Ring1a, led to an increased number of AP + colonies compared with the control shRNA group (Fig. 7a). These results, showing increased number of AP + colonies after Cbx4 or Cbx6 knockdown, were similar to the increased number of AP + colonies after Usp26 knockdown (Fig. 1c), suggesting that Cbx4 or Cbx6 inhibits cellular reprogramming. Sox2 and Nanog mRNA levels, but not Oct4, dramatically increased in Cbx4 or Cbx6 knockdown cells (Fig. 7b), similar to the increased Sox2 and Nanog mRNA levels in Usp26 knockdown cells (Fig. 3a). To determine the reprogramming efficiency of Usp26 knockdown alone, and with different combinations of Usp26 with Cbx4 and/or Cbx6 shRNAs, we performed similar experiments and found no significant differences in reprogramming efficiency between Usp26 knockdown alone and different combinations of Usp26 with Cbx4 and/or Cbx6 shRNAs. Furthermore, Cbx4 or Cbx6 alone could inhibit increased reprogramming efficiency mediated by Usp26 shRNA (Supplementary Fig. 6b). We also showed that Cbx4 and Cbx6 could promote ESC differentiation in Usp26 knockout cells (Supplementary Fig. 6c), similar to results obtained by overexpressing Usp26 (Fig. 2g). Overall, these results indicate that USP26 regulates CBX4 and CBX6 protein abundance through its deubiquitination activity during ESC differentiation, the increased amounts of CBX4 and CBX6 inhibits Sox2 and Nanog expression, thus blocking cellular reprogramming and reducing pluripotency.
Discussion

The development of strategies to more efficiently reprogram somatic cells into a pluripotent state has broader applicability for disease modeling and tissue regeneration. The epigenetic modifier PRC1 is directly involved in regulating gene expression. The components of PRC1 complexes are more diverse than PRC2 complexes, which primarily function as H3K27-specific histone methyltransferases. PRC1 complexes mediate the mono-ubiquitination of histone H2A to repress transcriptional elongation for maintaining ES pluripotency. For instance, USP family member USP28 regulates the stability of Myc protein, which is a key factor for somatic cell reprogramming. Another USP family member, USP22, acts as a transcriptional repressor of the Sox2 locus during ESC differentiation, implicating its role in adult somatic stem cell potency. Furthermore, the USP family member USP16 can remove Ub from H2AK119 and is required for ESC differentiation. In addition, USP7 and USP11 can regulate the ubiquitination status of PCGF2 and BMI1 in primary human fibroblast cells, leading to derepression of INK4A. Although these studies link USP proteins to sustaining pluripotency, the role of USPs in the regulation of the components of PRC1 complexes during cellular reprogramming has never been clearly elucidated.

Using screening of shRNA specific for the DUB subfamily USPs, we identified a novel cellular reprogramming repressor gene Usp26, which blocked somatic cell reprogramming into
iPSCs and promoted differentiation by stabilizing PRC1 complexes. A major component of the canonical PRC1 complexes is CBX7, which is present within the complex while ESCs are in a pluripotent state. Our results demonstrate that USP26 can diminish the expression of the Cbx7 gene and inhibit cellular reprogramming. Furthermore, the expression of CBX7 protein did not increase during ESC differentiation in contrast to the increase in CBX4 and CBX6 protein expression. Our finding is concordant with the notion that the binding of different components of the PRC1 complex acts as a switch between maintenance of pluripotency and differentiation.

In fact, a previous report revealed that CBX4 acted to repress the expression of Cbx7. In line with this observation, we further demonstrated that CBX4 and CBX6 can bind to the promoter of Cbx7. Conversely, lesser extent of CBX7 protein binding to the promoters of Cbx4 and Cbx6 is observed during USP26-mediated ESC differentiation and RA-induced ESC differentiation. We further identified that USP26 directly bound to CBX4 and CBX6 proteins and other components of the PRC1.2 complex, including RING1A, PCGF2, and CBX7. However, the interaction of USP26 with CBX4 and CBX6 was independent of RING1A. In differentiated cells, USP26 removed K48-linked Ub chains from CBX4 and CBX6, thus stabilizing the proteins and preventing their degradation. Henceforth, the accumulation of CBX4 and CBX6 proteins increased pluripotent gene (Sox2 and Nanog) and PRC1 gene (Cbx7) promoter occupancy and decreased promoter activity. The knockdown of Cbx4 or Cbx6 during OSKM-induced MEF reprogramming led to increased numbers of AP+ colonies and increased pluripotent gene (Sox2 and Nanog) expression, which is consistent with the improved reprogramming efficiency obtained with the knockdown of USP26. However, USP26 shRNA combined with Cbx4 or Cbx6 shRNA in OSKM-induced reprogrammed MEFs did not further increase numbers of AP+ colonies, suggesting that USP26 regulates somatic cell reprogramming through the control of CBX4 and CBX6 protein abundance, which in turn inhibit cellular reprogramming.

The knockdown of USP26 does not further increase Oct4 gene expression during OSKM-induced MEF reprogramming, but the ectopic expression of USP26 in ESCs results in the reduction of the expression of all three pluripotency genes, suggesting that a low level of Oct4 expression is sufficient to maintain ESC in a stable pluripotent state. Correlative with the Oct4 gene expression data, occupancy at the Oct4 promoter by USP26, CBX4, CBX6, and H2A-ub1 was low even during USP26-mediated ESC differentiation. However, H3K27me3 levels dramatically increased at the Oct4 promoter, which was similar to previously published reports. While our data show that USP26 does not directly bind to proteins of other PRC1 complex components, such as RING1B, RYBP, PCGF1, BMI1, KDM2B, and PHC1, these complexes may still bind to the Oct4 promoter...
to induce USP26-independent Oct4 gene expression. Therefore, the increased H3K27me3 levels at the Oct4 promoter might be due to USP26-independent recruitment of PRC2.

In summary, our study identifies an important role for USP26 in cellular reprogramming by stabilizing two core components CBX4 and CBX6 of the PRC1.2 complex. Mechanistically, the availability of the accumulated CBX4 and CBX6 facilitates their binding to the promoters of the pluripotent genes and may lead to the observed inhibition of pluripotent gene expression as well as the switching of the different components of PRC1.2. As a broader perspective, the greater understanding of the regulation of these transcription factors and epigenetic modifiers in cellular reprogramming is warranted for the rapid and consistent iPSC generation.

Methods

**MCF cell isolation.** Animal experiments were performed in accordance with an approved protocol from the Institutional Animal Care and Use committee (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute). Kill a pregnant OSKM mouse on 13 or 14 day post coitum by cervical dislocation. Dissect out the uterine horns and (Houston Methodist Research Institute).

**Cell culture.** Human embryonic kidney (HEK) 293T cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) with 10% fetal bovine serum (Valley Biomedical) and 1% antibiotic–antimycotic solution (Gibco). ESCs were maintained in ES culture medium (DMEM supplemented with 10% Knockout serum, 2 mM L-glutamine, 100 μM non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol, and 50 ng ml⁻¹ LIF). For USP26-induced differentiation, ES cells were transfected with lentiviruses expressing Dox-inducible GFP-tagged Usp26 or empty vector (Day – 1). After ESCs formed colonies, 0.1 μg ml⁻¹ Dox was added for pre-selection (Day – 1). Day 0 was defined as the day when pre-selected GFP-positive colonies were cultured in iSF1 medium with high-dose Dox (2 μg ml⁻¹), and individual colonies were tracked and taken pictures on days 1, 2, 3, and 4. For RA-induced differentiation, Usp26 KO, Cbx4 KO, Cbx6 KO, or WT ES cells were cultured in ES differentiation medium (DMEM supplemented with 10% Knockout serum, 2 mM L-glutamine, 100 μM non-essential amino acids (Gibco), and 0.1 mM β-mercaptoethanol) with 1 μM RA. Individual colonies were tracked and photographed over 8 days.

**Generation and reprogramming efficiency evaluation of iPSCs.** Mouse iPSCs were generated from MEFs with some minor modifications. Tet-O-inducible OSKM MEFs were used to generate iPSCs. OSKM transgenic MEF cells were transduced with GIPZ lentivirus-based shRNAs targeting USP family members, Cbx4, Cbx6, or Cbx7, and Cbx6. These cells were selected with 2 μg ml⁻¹ puromycin for 2 days. Before reprogramming, 3 x 10⁵ feeder cells (irradiated MEFs) per well were seeded into six-well plates previously coated with 0.1% gelatin. Thousand puromycin-selected OSKM transgenic MEF cells were reseeded onto feeder cells in six-well plates. The next day, modified iSF1 medium (DMEM supplemented with 10% Knockout serum, 2 mM L-glutamine, 100 μM non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol, 50 ng ml⁻¹ LIF, 5 μg ml⁻¹ CHIR99021, and 2.5 μg/ml PD0325901) containing 2 μg ml⁻¹ Dox was added and replenished every day. The efficiency of iPSC formation was calculated based on the number of AP⁺ iPSC colonies. The colonies were stained for AP activity on day 12 with AP detection Red Substrate kit (Vector).

**Teratoma assay.** iPSCs (1 x 10⁶) were injected subcutaneously into the skin on the dorsal rear flank of five severe combined immunodeficiency (SCID) mice (6 week, female). Four weeks after injection, mice were killed and tumors were removed and

![Image](https://example.com/image.png)
fixed in formalin for 24 h. These tumors were imbedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological analysis.

**Immunostaining.** To fully characterize iPS cells, these cells were fixed with methanol for 20 min at −20 °C, and nonspecific receptors were blocked with 10% normal goat serum. Cells in culture plates or chamber slides were fixed for 20 min at −20 °C with methanol, and nonspecific receptors were blocked with 10% normal goat serum. Oct4, SSEA1, and Nanog were stained with specific antibodies (anti-Oct4, 1:500, Santa Cruz Biotechnology SC-5279; anti-Nanog, 1:500, Santa Cruz Biotechnology SC-8178; and anti-Dppa4, 1:100, Cell Signaling 62576; anti-SSEA1, 1:1000, Invitrogen MA1-022), followed by goat anti-mouse antibody-conjugated serum. Oct4, SSEA1, and Nanog were stained with specific antibodies. Crosslinking was reversed by overnight incubation at 65 °C in reverse crosslink dithiothreitol (DTT). Bound complexes were eluted from the beads, and shear crosslinked DNA, with a minor modification of 4 for 12 s pulses (30 s pause between pulses) at 4 °C. The resulting whole-cell extract was pre-cleared with 50 μl protein A/G beads, 10 μl IgG, 10 μl 5% bovine serum albumin (BSA), and 5 μg of sheared salmon sperm DNA for each sample. After centrifugation, 20% of the supernatant was incubated overnight at 4 °C with 30 μl of Protein A/G agarose beads and 3 μg of the appropriate antibody, the beads were washed four times with ChIP buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA (pH 8.0), 150 mM NaCl, and 20 mM Tris-HCl (pH 8.0)) and once with trim-EDTA (TE) containing 1 mM dithiothreitol (DTT). Bound complexes were eluted from the beads, and crosslinking was reversed by overnight incubation at 65 °C in reverse crosslink buffer (1% SDS, 100 mM NaHCO3, 1 μg/ml RNase A, and 500 mM NaCl).

Whole-cell extract DNA was also treated for reverse crosslinking. Immunoprecipitated DNA and whole-cell extract DNA were purified by Zymoclean PCR purification kit (Zymo). ChIP–PCR primers are listed in Supplementary Table 1. Mouse ESCs were grown to 80–90% confluence and were chemically crosslinked by the addition of fresh formaldehyde solution (37%) to a final concentration of 1% for 10 min at room temperature. Cells were rinsed twice with cold PBS followed by addition of 2 M glycine to stop crosslinking and were collected using a silicon scraper. Cells were lysed and sonicated to solubilize and shear crosslinked DNA, with a minor modification of 4 for 12 s pulses (30 s pause between pulses) at 4 °C. The resulting whole-cell extract was pre-cleared with 50 μl protein A/G beads, 10 μl IgG, 10 μl 5% bovine serum albumin (BSA), and 5 μg of sheared salmon sperm DNA for each sample. After centrifugation, 20% of the supernatant was incubated overnight at 4 °C with 30 μl of Protein A/G agarose beads and 3 μg of the appropriate antibody, the beads were washed four times with ChIP buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA (pH 8.0), 150 mM NaCl, and 20 mM Tris-HCl (pH 8.0)) and once with trim-EDTA (TE) containing 1 mM dithiothreitol (DTT). Bound complexes were eluted from the beads, and crosslinking was reversed by overnight incubation at 65 °C in reverse crosslink buffer (1% SDS, 100 mM NaHCO3, 1 μg/ml RNase A, and 500 mM NaCl).

Whole-cell extract DNA was also treated for reverse crosslinking. Immunoprecipitated DNA and whole-cell extract DNA were purified by Zymoclean PCR purification kit (Zymo). ChIP–PCR primers are listed in Supplementary Table 1. Antibodies used for ChIP, western blotting, and immunoprecipitation were purchased using the Zymoclean™ Gel DNA Recovery Kits (Zymo) and sequenced. Sequencing results were analyzed with TIDE web tool (https://tide.nki.nl).

**IP and western blotting.** For IP, whole-cell extracts were prepared after transfection or stimulation with appropriate ligands, followed by incubation overnight with the appropriate antibodies plus protein A/G beads (Pierce). Beads were washed five times with low-salt lysis buffer, and immunoprecipitates were eluted with 4× SDS loading buffer and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Bio-Rad) followed by further incubation with the appropriate antibodies. Luminata Crescendo Western HRP substrate (Millipore) was used for protein detection. For endogenous IP, mouse ESC nuclear extracts (150 μl for each IP) were immunoprecipitated with appropriate antibodies (3 μg for each IP) followed by western blotting or mass spectrometry analysis. As controls, either rabbit or mouse IgG antibodies were used. For interaction studies, 293T cells after transfection, cells were lysed in cell lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 2 mM MgCl2, U 8 benzamide, and 10% glycerol) with 1 μg/ml trypsin inhibitor maximized for 30 min, followed by IP of cell lysates with anti-FLAG M2 antibody (1:2000, Sigma F3165). Next, immunocomplex pulled down by anti-FLAG M2 antibody was subjected to western blotting with anti-RING1A, CBX4, CBX6, CBX7, and PCGF2 antibodies. As controls, whole-cell extracts were fractionated by SDS-PAGE, followed by immunoblotting with specific antibodies against the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132, 567–582 (2008).

**Plasmids constructs.** Full-length mouse Usp26, Cbx4, and Cbx6 cDNA were obtained from MEF cDNA by two-step PCR and cloned into pcDNA3.1 with HA tag sequence. A similar strategy was used to clone human USP26. The C-terminal GFP-tagged mUsp26, mCbx4, and mCbx6 cassettes were amplified by overlapping PCR and cloned into pTet-O lentiviral vector through BP and LR reactions of Gateway cloning system (Invitrogen). pTet-O-mUsp26-GFP, pTet-O-mCbx4-GFP, pTet-O-mCbx6-GFP, pTet-O-mCbx7-GFP, pcDNA-HA-hUSP26, and pcDNA-HA-hMUSP26 were sequenced to verify the correct DNA sequence and their open reading frames.

**Lentivirus production and transduction.** One day before transfection, HEK293T cells were seeded at 50% confluency in 15 cm dishes. Cells were transfected the next day at 80–90% confluency. For each flask, 20 μg of plasmid containing the vector of interest, 10 μg of VSV-G, and 15 μg of Δ8.9 were transfected using calcium transfection methods. Five hours (h) after transfection, the media was changed. Virus supernatant was harvested at 48 h post transfection, filtered with a 0.45 μm polyvinylidene fluoride (PVDF) filter (Millipore), and centrifuged at 25,000 × g for 2 h. Lentiviral pelus was resuspended with 1 ml target cell medium. MEFs were cultured in DMEM supplemented with 10% Knockout serum, 2 mM L-glutamine, 100 μM non-essential amino acids (Gibco), and β-Mercaptoethanol, and passaged every other day at a 1:4 ratios. Cells were transduced with lentivirus via spinfection in six-well plates. One thousand cells in 2 ml of media supplemented with 8 mg ml−1 polybrene (Sigma) were added to well supplemented with 10 μl lentiviral supernatant. Medium was refreshed on day 2, and cells were passaged every other day starting on day 4 after replating.

**CRISPR knockout.** Short guide RNAs (sgRNAs) were designed using the CRISPRTool (http://crispr.mit.edu) to minimize potential off-target effects. sgRNA products and genomic primers are listed in Supplementary Table 1. Corresponding oligonucleotides were ordered (IDT) and subcloned into the Lentivirus plasmid (Addgene), expressing a human codon-optimized SpCas9, guide RNA, and puromycin expression plasmid, following a previously published protocol34. Specific sgRNA lentiviruses were packaged as described in the Methods section. One milliliter lentiviral supernatant was added into 1 × 10^6 293T cells or 1 × 10^6 MEFs in six-well plates. After 48 h, 2 or 4 μM puromycin was used for selection. Puromycin-selected 293T or MEF cells (Fig. 4c and Supplementary Fig. 1b–e) or MEFs (Supplementary Fig. 2c, d) were expanded for western blot assay.

**TIDE assay.** Genomic regions surrounding sgRNA-targeted sites were amplified by PCR. TIDE assay35 primers are listed in Supplementary Table 1. PCR products were purified using the Zymoclean™ Gel DNA Recovery Kits (Zymo) and sequenced. Sequencing results were analyzed with TIDE web tool (https://tide.nki.nl).

**Deubiquitination activity assay.** FLAG-USP26 (21 μm) proteins were incubated with 100 ng of poly-linked Ub chains (K48 or K29, BIOMOL) for 3 h at 37 °C in 50 mM Tris (pH 8.0) and 1 mM DTT, separated by SDS-PAGE, and analyzed by anti-Ub immunoblotting (1:1000 dilution; Santa Cruz Biotechnology).

**Luciferase assay.** 293T cells (8 × 10^5 cells) were transduced with reporter (pLenti-Luc-OCT4, SOX2, NANOG, or CRX7) lentiviral supernatants and then transduced with 100 ng HA-USP26, HA-CBX4, or HA-CBX6. Cell lysate was prepared following harvesting cells 24 h after transfection, and reporter activity was measured with the Dual Luciferase Assay (Promega).

**Statistical analysis.** Significant differences between groups were assessed with two-way ANOVA test or two-tailed Student’s t-test. Values of p < 0.05 were considered statistically significant.

**Data availability.** The data sets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Received: 27 May 2016 Accepted: 20 June 2017
Published online: 24 August 2017

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Acknowledgements
This work was in part supported by grants from the NCI, NIH (1R01CA189032 and 1R01CA101795), Cancer Prevention and Research Institute of Texas (CPRIT), National Natural Science Foundation of China (No. 61572766), and Dottie and Jimmy C. Adair Myelodysplastic Syndrome Research and Treatment Fund. W.L. was supported in part by Xiangya Hospital, Xiangya School of Medicine, Central South University, China. We would like to thank Dr. Jana Burchfield for critical reading of this manuscript.

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Conceptualization: B.N. and R.-F.W.; methodology: W.Z., C.Q., Q.L., P.L., and W.L.; writing—original draft: B.N.; writing—review and editing: R.-F.W.; funding acquisition: R.-F.W.

Additional information
Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00301-4.

Competing interests: The authors declare no competing financial interests.

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