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UbE2E1/UBCH6 Is a Critical *in Vivo* E2 for the PRC1-catalyzed Ubiquitination of H2A at Lys-119*

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Keith Wheaton[‡], Feroz Sarkari[‡], Beena Stanly Johns[‡], Hossein Davarinejad[‡], Olga Egorova[‡], Lilia Kaustov[§], Brian Raught[§], Vivian Saridakis[‡], and Yi Sheng[‡1]

From the [‡]Department of Biology, York University, Toronto, Ontario M3J1P3 and the [§]Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario M5G 1L7, Canada

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UbE2E1/UbcH6 is an E2 ubiquitin-conjugating enzyme that is regulated by USP7. We identified UbE2E1 as a novel component of Polycomb repressive complex 1 (PRC1), the E3 ligase complex responsible for histone H2A ubiquitination and gene silencing. We demonstrate that UbE2E1 is critical for the monoubiquitination of H2A at residue Lys-119 (uH2AK119) through its association with the PRC1 complex. UbE2E1 interacts with PRC1 subunits including Ring1A and Ring1B. Overexpression of UbE2E1 results in increased levels of uH2AK119, whereas overexpression of catalytically inactive UbE2E1_C131A or UbE2E1 knockdown results in decreased levels of uH2AK119. The down-regulation of H2A ubiquitination by loss of function of UbE2E1 is correlated with alleviated p16[INK4a] promoter repression and induced growth inhibition in HCT116 cells. These results are specific to UbE2E1 as knockdown of UbE2D E2s does not show any effect on uH2AK119. We extended the UbE2E1 regulation of uH2AK119 to USP7 and showed that USP7 is also a key regulator for monoubiquitination at H2A Lys-119 as both knockdown and deletion of USP7 results in decreased levels of uH2AK119. This study reveals that UbE2E1 is an *in vivo* E2 for the PRC1 ligase complex and thus plays an important role in the regulation of H2A Lys-119 monoubiquitination.

Monoubiquitination of H2A at Lys-119 (uH2AK119) is found on 5~15% of total H2A in mammalian cells. H2A monoubiquitination plays an essential role in the maintenance of genome integrity and pluripotency of stem cells, developmental patterning, and X-chromosome inactivation (1–6). The monoubiquitination of H2A at residue Lys-119 is primarily mediated by Polycomb repressive complex 1 (PRC1),[2] which contains three RING domain proteins Ring1A (RING1), Ring1B (RING2 or RNF2), and one of the PCGF proteins. The core PRC1 proteins Ring1B and BMI1 (also known as PCGF4) form an active heterodimeric E3 ubiquitin ligase for monoubiquitination of H2A at Lys-119. Genetic studies revealed that Ring1B is the main catalytic subunit of the PRC1 complex as Ring1B knock-out leads to global loss of uH2A and embryonic lethality (4, 7, 8). However, BMI1 and Ring1A also play important roles in stimulating the E3 ligase activity and maintaining the integrity of the PRC1 complex. Deletion of BMI1 and Ring1A lead to a significant decrease of uH2AK119 levels and developmental abnormalities (3). Despite extensive studies characterizing the E3 ligases responsible for ubiquitination of H2A, little is known about the specific E2-conjugating enzyme(s) for H2A ubiquitination. Although several E2s including the UbE2D family (UbE2D1/UbcH5a, UbE2D2/UbcH5b, and UbE2D3/UbcH5c) and UbE2E1/UbcH6 have been reported to assist Ring1B•BMI1 in catalyzing monoubiquitination of H2A, these are all based on biochemical studies and no E2 has yet been identified for the PRC1 complex in a cellular context (9, 10). UbE2Ds and UbE2E1 share ~80% sequence identity within their catalytic ubiquitin-conjugating (UBC) domains. Distinct from UbE2D subfamily members, UbE2E1 contains a 50-residue unstructured N-terminal extension rich in serine and lysine residues, which has been shown to interact with USP7 and is essential for USP7-mediated UbE2E1 stabilization (11).

Several deubiquitinating enzymes (DUBs) including UBP-M/USP16, USP3, USP21, USP22, and 2A-DUB have been reported to act directly on uH2AK119 and influence its function. Specifically, UBP-M/USP16 is required for dynamic H2A deubiquitination during cell cycle progression (12, 13). USP21, USP22, and 2A-DUB function as positive transcriptional regulators via deubiquitination of H2A (14–16). USP3 is involved in the DNA damage repair pathway and deubiquitinates H2A among other substrates in DNA damage foci (17). In addition, DUBs can indirectly regulate the levels of uH2AK119 by affecting the function and stability of enzymes involved in H2A ubiquitination. For example, USP7 and USP11 were purified with the PRC1 complex and shown to modulate its transcriptional regulation of the *INK4a/ARF* locus (18). Also USP7 has been identified as an interacting partner of Ring1B in separate studies indicating its involvement in PRC1 function (18–20). More recently, USP7 was identified to regulate PRC1 function via its association with SCML2 (21).

H2A monoubiquitination at Lys-119 has been associated with gene silencing of the *INK4A/ARF* locus in the maintenance of stem cell proliferative activity and the sustainment of unlim-

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[1] To whom correspondence should be addressed. E-mail: yisheng@yorku.ca.

[2] The abbreviations used are: PRC1, Polycomb repressive complex 1; UBC, ubiquitin-conjugating; DUB, deubiquitinating enzyme; IP, immunoprecipitation; PCGF, polycomb group ring finger.

A

Selected UbE2E1 interacting proteins identified by affinity capture MS

Gene ID	Interacting proteins	Number of Peptides Identified
7324	UbE2E1	1215
7874	USP7	36
55611	OTUB1	30
6015	Ring1A	18
84333	PCGF5	15
3015	H2A.Z	29
9555	macroH2A	6
3005	Histone H1	13

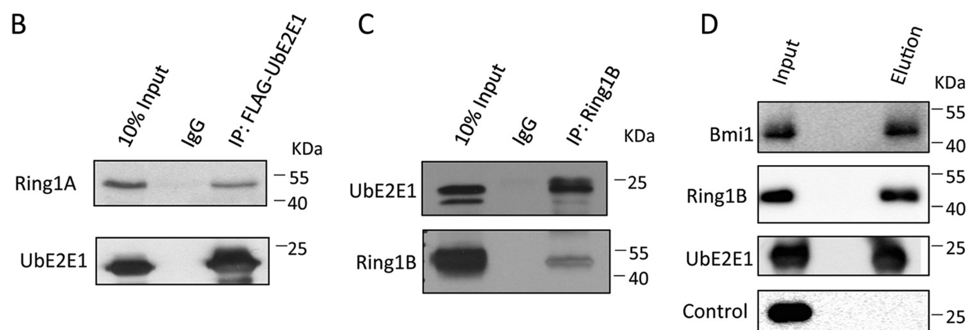

FIGURE 1. **UbE2E1 interacts with PRC1.** *A,* UbE2E1 interaction partners identified using affinity capture proteomic studies. *B,* UbE2E1 interacts with Ring1A. FLAG-tagged UbE2E1 was expressed in U2OS cells and immunoprecipitated with an antibody against the FLAG tag, followed by Western blotting using antibodies against Ring1A and UbE2E1. *C,* UbE2E1 interacts with Ring1B. U2OS cell lysates were immunoprecipitated with a Ring1B antibody followed by Western blotting with antibodies against endogenous Ring1B and UbE2E1. *D,* FLAG pulldown of UbE2E1 with FLAG-tagged Ring1B·BMI1. UbE2E1 was incubated with FLAG-Ring1B·BMI1-captured complex and eluted with FLAG-peptide followed by Western blotting with antibodies against Ring1B, BMI1, UbE2E1, and GST as negative control.

ited cell proliferation in cancer cells (22–24). Deletion or transcription repression of the *INK4A*/*ARF* locus are common in many malignant cancers. The *INK4A*/*ARF* locus encodes two tumor suppressor proteins $p16^{INK4a}$ and $p14^{ARF}$. The $p16^{INK4a}$ protein inhibits the cyclin-dependent kinase (CDK4 and CDK6) complexes by blocking cyclin D association, thereby negatively regulating cell cycle progression and cell proliferation. The $p14^{ARF}$ protein enhances the function of tumor suppressor p53 and promotes apoptosis and senescence in response to oncogenic stresses. Activation of $p16^{INK4a}$ and $p14^{ARF}$ transcription leads to cell cycle arrest and cellular senescence, whereas deletion or repression of this gene locus results in uncontrolled cell growth and transformation (reviewed in Ref. 25). Importantly, the levels of uH2AK119 appear to be correlated with transcriptional silencing of this locus. Loss of uH2AK119 at the promoter leads to de-repression of the *INK4A*/*ARF* locus, whereas enriched uH2AK119 results in repression of this locus (26). The level of uH2AK119 is tightly regulated and critical in determining the cellular activity of $p16^{INK4a}$ and $p14^{ARF}$.

In this study, we explored novel UbE2E1 interacting proteins and found that UbE2E1 interacts with components of the PRC1 complex. We further demonstrated that UbE2E1 is critical for PRC1-mediated monoubiquitination of H2A Lys-119. Through the regulation of the levels of uH2AK119, UbE2E1 influences transcriptional silencing of the *INK4A*/*ARF* locus. Our study revealed that UbE2E1 regulates H2A ubiquitination by functioning as an endogenous E2 for PRC1-mediated H2A ubiquitination. Thus, UbE2E1 plays a critical role in homeostasis of H2A ubiquitination.

Results

UbE2E1 Binds Components of the PRC1 Complex—E2 enzymes are critical components of the protein ubiquitination machinery as they influence E3 ligase activity and modulate different types of ubiquitin modification (27). We performed affinity capture LC-MS/MS proteomic studies, using overexpressed FLAG-tagged UbE2E1 as bait, to identify UbE2E1 interacting proteins in U2OS cells. PRC1 proteins Ring1A and PCGF5 were identified as novel UbE2E1 interacting proteins (Fig. 1*A*). In addition, USP7 was also captured as a binding partner of UbE2E1, and was shown to be critical in controlling UbE2E1 stability (11).

To address whether UbE2E1 associates with components of the PRC1 complex, we performed co-immunoprecipitation analyses to test the interaction between UbE2E1 and Ring1A or Ring1B. Consistent with the MS data, endogenous Ring1A was readily pulled down with FLAG-UbE2E1 (Fig. 1*B*). Although Ring1B, the main E3 ligase of the PRC1 complex was not detected in the initial UbE2E1 proteomic study, the interaction between Ring1B and UbE2E1 was confirmed by immunoprecipitation using an antibody against endogenous Ring1B and immunoblotting for endogenous UbE2E1 (Fig. 1*C*). Together, these results suggest that UbE2E1 interacts with components of the PRC1 complex *in vivo*.

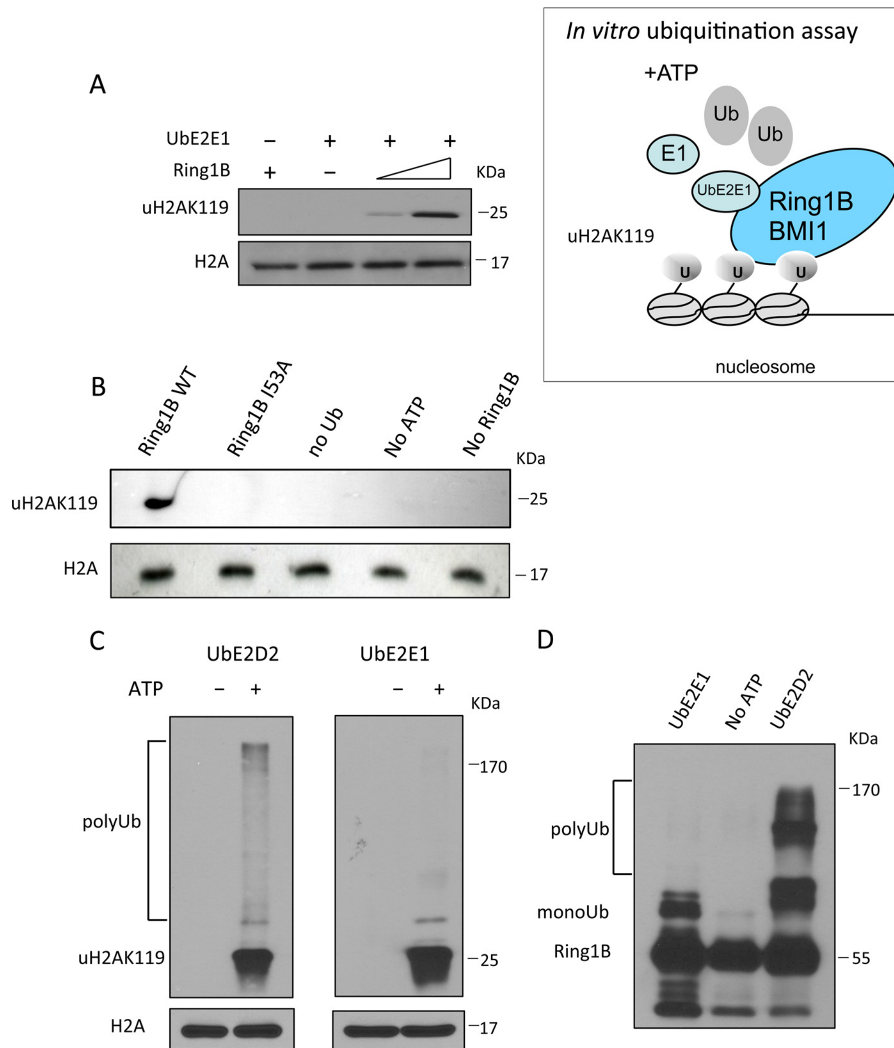

FIGURE 2. **UbE2E1 modulates PRC1 catalyzed mono-ubiquitination of H2A.** *A, in vitro* ubiquitination of H2A using reconstituted nucleosome as substrate in the presence or absence of UbE2E1 and Ring1B·BMI1 complex. In each reaction, equal amounts of Ub, E1, and nucleosome were added. Reactions were analyzed by Western blotting using antibodies against total H2A or uH2AK119. *B, in vitro* ubiquitination of H2A using reconstituted nucleosome as substrate in the presence of wild-type or mutant Ring1B. Reactions were analyzed by Western blotting using antibodies against total H2A or uH2AK119. *C, in vitro* ubiquitination of H2A using reconstituted nucleosome as substrate in the presence of UbE2E1 or UbE2D2. Reactions were analyzed by Western blotting using antibodies against total H2A or uH2AK119. *D, in vitro* autoubiquitination of Ring1B in the presence of UbE2E1 or UbE2D2. Reactions were analyzed by Western blotting using a Ring1B antibody.

An *in vitro* pulldown assay was used to demonstrate that UbE2E1 interacted directly with the FLAG-Ring1B·BMI1 dimer. FLAG-Ring1B·BMI1 was overexpressed in U2OS cells and immobilized on FLAG-agarose. UbE2E1 or GST as negative control was then incubated with the Ring1B·BMI1 FLAG-agarose. UbE2E1 but not GST was co-eluted with the Ring1B·BMI1 heterodimer indicating that UbE2E1 interacted with the Ring1B·BMI complex (Fig. 1*D*).

UbE2E1 Is Critical for H2A Monoubiquitination—To test whether UbE2E1 is an E2 for Ring1B·BMI1-catalyzed monoubiquitination of H2A, an *in vitro* ubiquitination assay was performed using UbE2E1, FLAG-Ring1B·BMI1, and reconstituted nucleosome as a substrate. The active Ring1B·BMI1 complex was purified from cell lysate using FLAG-agarose and eluted using a 3× FLAG peptide. Monoubiquitination of H2A was detected in the presence of both UbE2E1 and Ring1B·BMI1 but not either alone, indicating that UbE2E1 can act as the E2 for PRC1-mediated H2A monoubiquitination (Fig. 2*A*). To test whether the ubiquitination of H2A Lys-119 in the presence of UbE2E1 was specific for the PRC1 complex, an inactive Ring1B mutant (I53A) was prepared. This I53A Ring1B mutant was unable to catalyze the monoubiquitination of H2A Lys-119 (Fig. 2*B*). These results are consistent with other studies showing that UbE2E1 is involved in the monoubiquitination of H2A catalyzed by recombinant Ring1B·BMI1 *in vitro* (9). Previous studies have shown that UbE2D family proteins assist Ring1B·BMI1 to monoubiquitinate H2A at Lys-119 (9, 10, 28, 29). To compare the function of UbE2E1 and UbE2D proteins, we examined Ring1B·BMI1-mediated H2A ubiquitination and Ring1B autoubiquitination. Both UbE2E1 and UbE2D2 are active in Ring1B·BMI1-mediated H2A ubiquitination at Lys-119. As shown in Fig. 2*C*, UbE2E1-Ring1B·BMI1 catalyzes only H2A Lys-119 monoubiquitination, whereas UbE2D2-Ring1B·BMI1 carries out both monoubiquitination at H2A Lys-119 and polyubiquitination of H2A. These results suggest that UbE2E1 and UbE2D proteins have distinct functions in H2A ubiquitination. Interestingly, Ring1B was autoubiquitinated in these *in vitro* H2A ubiquitination reactions (Fig. 2*D*). In the

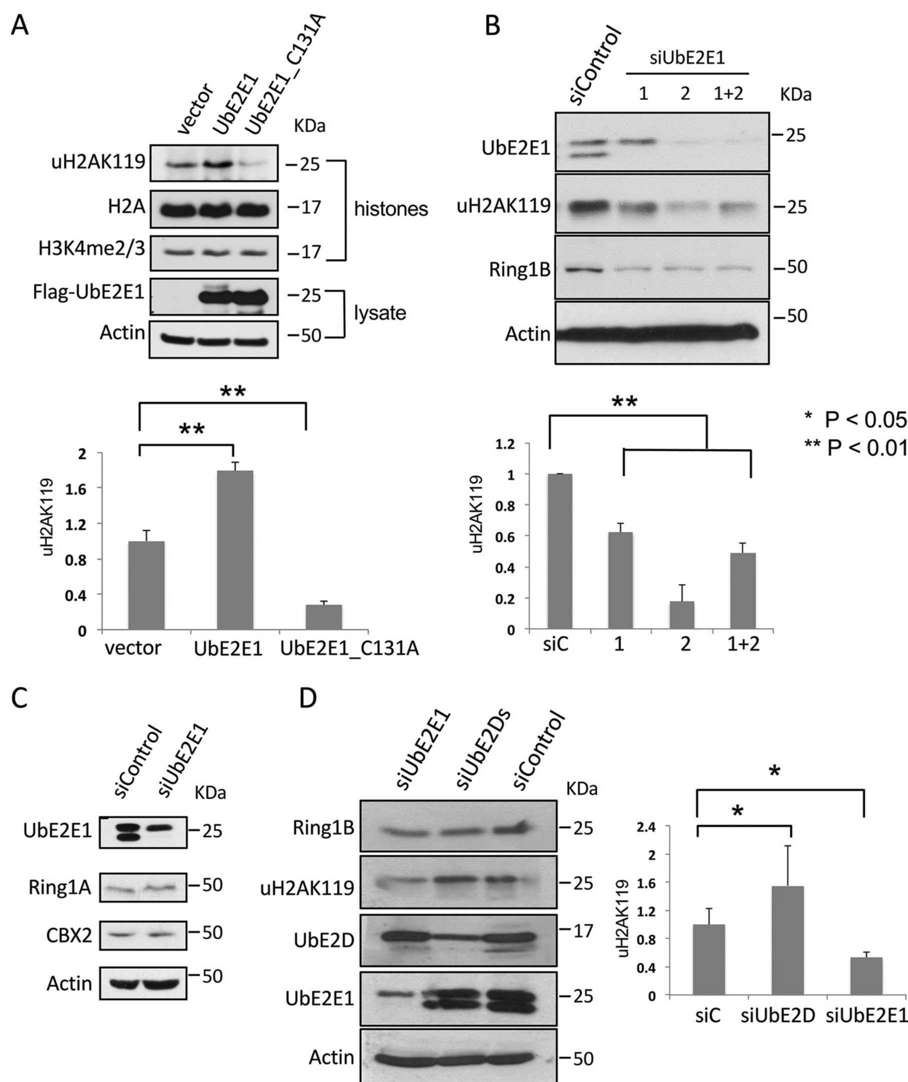

FIGURE 3. **UbE2E1 is critical for H2A monoubiquitination.** *A,* overexpression of UbE2E1 or UbE2E1_C131A. HCT116 cells were transfected with empty vector, FLAG-tagged UbE2E1, or UbE2E1_C131A. Total histones or cell lysates were immunoblotted using the indicated antibodies. *B,* knockdown of UbE2E1. U2OS cells were transfected with nonspecific control siRNA (siControl) or siRNAs targeting UbE2E1 (siUbE2E1 #1, siUbE2E1 #2, or combination of siUbE2E1 #1 and siUbE2E1 #2). Cell lysates were immunoblotted using the indicated antibodies. *C,* knockdown of UbE2E1. U2OS cells were transfected with nonspecific control siRNA (siControl) or an siRNA targeting UbE2E1. Cell lysates were immunoblotted using the indicated antibodies. *D,* knockdown of UbE2E1 and UbE2Ds in U2OS cells. U2OS cells were transfected with nonspecific control siRNA or siRNAs targeting UbE2E1 or UbE2Ds. Cell lysates were immunoblotted using the indicated antibodies. Fold-change of uH2AK119 was determined by normalizing uH2AK119 to actin. A paired *t* test was performed to evaluate statistical significance of the changes in uH2AK119 levels. *Error bars* indicate standard deviation and are from at least 2 lysates.

UbE2E1-specific assays, monoubiquitinated Ring1B was detected, whereas polyubiquitination of Ring1B was detected in the reactions using UbE2D2.

To gain better insight into the function of UbE2E1 in the PRC1 complex, we overexpressed UbE2E1 and the enzymatically inactive mutant UbE2E1_C131A. Ectopic UbE2E1 resulted in increased cellular levels of uH2AK119, but no change in total H2A or di- and trimethylated H3K4 (Fig. 3*A*). Ectopic UbE2E1_C131A, the enzymatically inactive mutant, resulted in decreased cellular levels of uH2AK119 with no effect on total H2A or di- and trimethylated H3K4 (Fig. 3*A*). Together, these results support the role of UbE2E1 in the PRC1-catalyzed monoubiquitination of H2A Lys-119. To further confirm the role of UbE2E1 in the PRC1 complex, two different siRNAs were used to knockdown UbE2E1. Both siRNAs or a combination of the two dramatically reduced endogenous UbE2E1 and resulted in decreased levels of uH2AK119. These results further support that UbE2E1 is an active E2 for H2A monoubiquitination at Lys-119 *in vivo*. Interestingly, knockdown of UbE2E1 also decreased the protein levels of Ring1B but not CBX2 or Ring1A (Fig. 3, *B* and *C*), indicating that UbE2E1 may also have a role in the stability of the main PRC1 E3 ligase, Ring1B.

To better understand the specificity of E2s for the monoubiquitination of H2A Lys-119 *in vivo*, we also examined knockdown of UbE2Ds. Surprisingly, the knockdown of UbE2Ds did not have any effects on the levels of either uH2AK119 or Ring1B (Fig. 3*D*). These results demonstrate that the UbE2Ds do not play an essential role in monoubiquitination of H2A Lys-119 *in vivo*.

USP7 Regulates the Levels of uH2AK119—USP7 was previously shown to directly interact with and stabilize UbE2E1 (11).

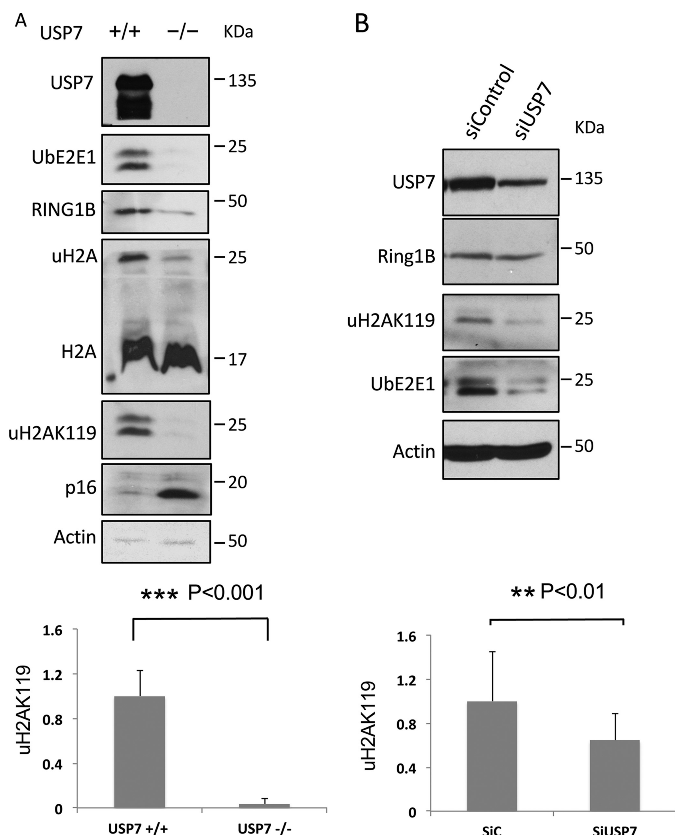

FIGURE 4. **USP7 regulates PRC1 and H2A monoubiquitination.** *A,* the lysates from HCT116 USP7$^{+/+}$ and USP7$^{-/-}$ cells were subject to immunoblotting using antibodies as indicated. *B,* USP7 knockdown was performed in HeLa cells by transfection with a USP7-specific siRNA. The lysates from USP7 knockdown or control HeLa cells were subject to immunoblotting using antibodies as indicated. Fold-change of uH2AK119 was determined by normalizing uH2AK119 to actin. A paired *t* test was performed to evaluate statistical significance of the changes in uH2AK119 levels. *Error bars* indicate S.D. and are from at least 2 lysates.

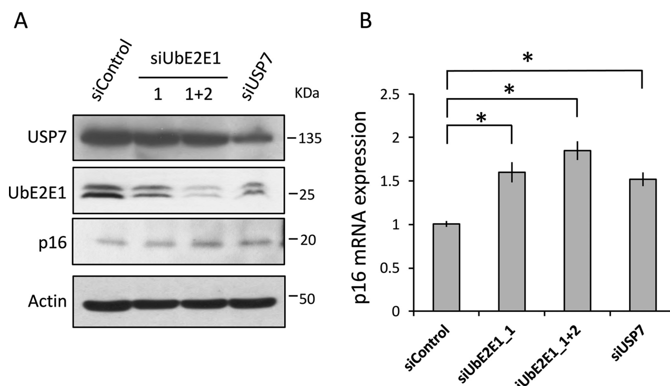

FIGURE 5. **UbE2E1 and USP7 regulate the levels of p16^{INK4a} mRNA and protein levels.** *A,* knockdown of UbE2E1 and USP7 in HeLa cells. HeLa cells were transfected with nonspecific control siRNA (siControl) or siRNAs targeting UbE2E1 (siUbE2E1 #1, or combination of siUbE2E1 #1 and siUbE2E1 #2) or USP7. Cell lysates were immunoblotted using the indicated antibodies. *B,* p16^{INK4a} mRNA expression levels were affected by UbE2E1 or USP7 knockdown. p16^{INK4a} mRNA expression was measured by quantitative RT-PCR on three independent biological replicates, normalized with an internal GAPDH control, and presented as fold-change over control. A paired *t* test was used to evaluate the differences between siUbE2E1 and siControl and between siUSP7 and siControl. The significance was denoted as *, $p < 0.01$.

USP7 was also previously shown to associate with chromatin, regulate the stability of the PRC1 complex, and deubiquitinate Ring1B (18, 20). However, the effect of USP7 on H2A monoubiquitination was not investigated in USP7 knock-out cells. Therefore, we examined levels of uH2AK119 in HCT116 USP7$^{-/-}$ cells in which the *USP7* gene is knocked out and also in HeLa cells with USP7 knockdown. Decreased levels of UbE2E1, Ring1B, and uH2AK119 were observed in USP7$^{-/-}$ compared with USP7$^{+/+}$ cells (Fig. 4*A*). Similarly, USP7 knockdown resulted in decreased levels of UbE2E1, Ring1B, and uH2AK119 (Fig. 4*B*). Thus, USP7 is a key regulator as it stabilizes components of PRC1, maintains the function of the PRC1 complex, and regulates the level of uH2AK119.

USP7 and UbE2E1 Contribute to Gene Silencing of the INK4A/ARF Locus—H2A Lys-119 monoubiquitination leads to transcriptional repression of the *INK4A/ARF* locus (18, 30). Because both USP7 and UbE2E1 modulate the level of uH2AK119, we examined the role of USP7 and UbE2E1 in transcriptional regulation of the *INK4A/ARF* locus. Knockdown of either UbE2E1 or USP7 resulted in an increase in p16^{INK4A} protein and mRNA levels (Fig. 5). It is known that p16^{INK4a} plays a critical role in the induction of cellular senescence (31). Knockdown of USP7 has also been reported to induce a senescence-like proliferative arrest phenotype (18). This led us to examine the levels of senescence in the *USP7* knock-out cells. There were increased numbers of senescent cells, as indicated by senescence-associated β-galactosidase staining in HCT116 USP7$^{-/-}$ cells (18 $\pm$ 4%) compared with parental HCT116 USP7$^{+/+}$ cells (none detected) (Fig. 6*A*), correlated with decreased H2A monoubiquitination and elevated level of p16^{INK4a} detected in these cells (Fig. 4*A*). To further demonstrate the role of p16^{INK4a} in the growth defect observed in HCT116 USP7$^{-/-}$ cells, we examined the effect of silencing p16^{INK4a} in these cells. In parental HCT116 USP7$^{+/+}$ cells, the level of p16^{INK4a} is quite low, therefore silencing p16^{INK4a} did not have any effect on the growth rate (Fig. 6, *B* and *D*, USP7$^{+/+}$ con siRNA *versus* USP7$^{+/+}$ p16 siRNA). However, silencing of p16^{INK4a} in HCT116 USP7$^{-/-}$ cells shows increased growth rate compared with the control siRNA cells, demonstrating that the growth defect of USP7$^{-/-}$ cells is at least in part due to the increased expression of p16^{INK4a} (Fig. 6, *B* and *D*, USP7$^{-/-}$ con siRNA *versus* USP7$^{-/-}$ p16 siRNA). Consistent with the increased p16^{INK4a} levels shown with UbE2E1_C131A overexpression (Fig. 6*C*), altered p16^{INK4a} function was demonstrated as a slower growth rate in USP7$^{+/+}$ cells transfected with UbE2E1_C131A (Fig. 6*E*, USP7$^{+/+}$ UbE2E1 *versus* USP7$^{+/+}$ UbE2E1_C131A). Together these data support the role of USP7 and UbE2E1 in determining the onset of senescence and cell growth by regulating the level of p16^{INK4a}, which is correlated with the role of USP7 and UbE2E1 in the regulation of uH2AK119.

Last, to confirm that the increased level of p16^{INK4a} following UbE2E1 knockdown was due to transcriptional de-repression mediated by uH2AK119, a native chromatin IP was performed in UbE2E1 knockdown and control cells using an antibody against uH2AK119. Three sets of primers targeting the *INK4A/ARF* locus at specific promoter regions of *INK4A* were used in a quantitative PCR experiment to examine the occupancy of ubiquitinated H2A at these regions. UbE2E1 knockdown showed 2–4-fold decreases for all three sites within the p16^{INK4a} promoter compared with the control (Fig. 6*F*). These results strongly imply

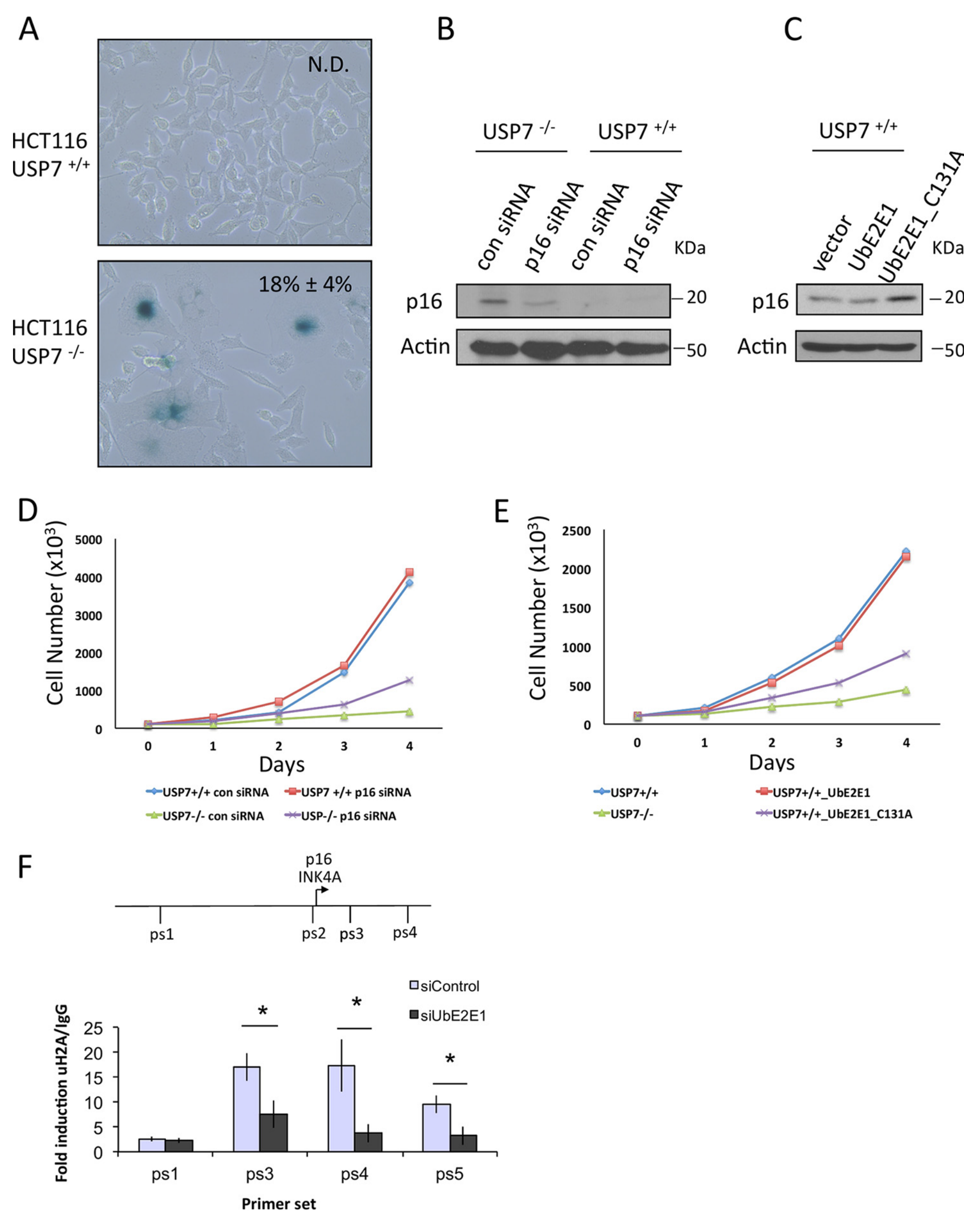

FIGURE 6. **Loss of USP7 leads to decreased H2A monoubiquitination, decreased growth rate, and increased senescence.** *A*, phase-contrast images showing senescence-associated *β*-galactosidase staining of HCT116 USP7$^{+/+}$ and USP7$^{-/-}$ cells. *Blue* staining is indicative of senescent cells. *B*, knockdown of p16^{INK4a} in USP7$^{-/-}$ cells. HCT116 USP7$^{+/+}$ and USP7$^{-/-}$ were transfected with siControl or siRNA against p16. Lysates were immunoblotted with a p16^{INK4a}-specific antibody. *C*, overexpression of UbE2E1 or UbE2E1_C131A. Levels of p16^{INK4a} increased in the presence of overexpressed UbE2E1_C131A. Lysates were immunoblotted with a p16^{INK4a}-specific antibody. *D*, cell growth of HCT116 USP7$^{+/+}$ and USP7$^{-/-}$ cells transfected with siControl or siRNA against p16^{INK4a} were monitored and plotted for 5 days. *E*, the effect of UbE2E1 on cell growth of HCT116 cells. HCT116 USP7$^{+/+}$ cells transfected with UbE2E1 or UbE2E1_C131A were monitored and compared with HCT116 USP7$^{-/-}$ cells. *F*, U2OS cells were transfected with control siRNA or siRNA targeting UbE2E1 and subject to ChIP using an antibody against uH2AK119. Quantitative PCR was performed using primer sets targeting different sites (ps1–4) within the *INK4A/ARF* locus as illustrated. The amount of uH2AK119 at different locations of *INK4A/ARF* locus was normalized by the background signal obtained using IgG and is represented by fold-induction of uH2A over IgG. All amplifications were performed in triplicate, and *error bars* represent S.D. A paired *t* test was used to evaluate the difference between siUbE2E1 and siControl. The significance was denoted as *, $p < 0.01$.

that UbE2E1 contributes to the transcriptional repression of *INK4A/ARF* by regulating monoubiquitination of H2A.

Discussion

In this study, we showed that both UbE2E1 and USP7 regulate the levels of uH2AK119. Importantly, UbE2E1 appears to be a critical E2 enzyme for *in vivo* PRC1-mediated H2A monoubiquitination. Knockdown of UbE2E1 or overexpression of the inactive UbE2E1_C131A mutant reduced the level of uH2AK119 accompanied by increased expression of its repressed target p16^{INK4a}. Similar effects have previously been observed by removing other subunits of the PRC1 complex (such as the E3 ligases, Ring1B, Ring1A, and BMI1) in various cell types with a suppressed p16^{INK4a} locus (23, 24, 26). These results imply that UbE2E1 is an integral component of the PRC1 complex and regulates the level of uH2AK119.

Previous *in vitro* studies have reported that more than one E2 can work with Ring1B·BMI1 to catalyze H2A Lys-119 ubiquitination (9, 10, 29). UbcH5c/UbE2D2 was shown to form a ternary complex with Ring1B·BMI1 and was active in catalyzing

Ring1B-dependent H2A ubiquitination (10). Recently, the crystal structure of the PRC1 complex including Ring1B•BMI1 and UbcH5d/UbE2D3 interacting with nucleosome revealed the importance of the E2 enzyme in stabilizing contact with the nucleosome (28). Nevertheless, several lines of evidence support UbE2E1 as a critical *in vivo* E2 for PRC1-mediated H2A Lys-119 monoubiquitination. UbE2E1 is located primarily in the nucleus (11) and our proteomic data shows that UbE2E1 interacts with components of the PRC1 complex. We were readily able to show co-immunoprecipitation of both Ring1B and Ring1A along with UBE2E1. As shown in this study, UbE2E1, but not UbE2Ds, functions in the ubiquitination of H2A Lys-119 *in vivo*, as knockdown of UbE2Ds did not result in changes in the levels of uH2AK119. This study also shows that UbE2Ds produce both mono- and polyubiquitin chains on H2A, whereas UbE2E1 only catalyzes monoubiquitination of H2A *in vitro*, which was also observed by Buchwald *et al.* (9). Because H2A can be modified by both polyubiquitination and monoubiquitination, UbE2D and UbE2E1 may have different roles in H2A ubiquitination, even though they can both assist with ubiquitination at H2A Lys-119. It is interesting to note that the UbE2E1 gene follows a similar evolutionary path to the Polycomb genes, which were initially identified in *Drosophila* and conserved in multicellular eukaryotes (32), whereas UbE2D genes are found in all eukaryotes. As uH2AK119 only occurs in multicellular eukaryotes, it is plausible that UbE2E1 and PRC1 co-evolved to catalyze this histone modification for gene silencing and transcriptional regulation (33). There are two other UbE2E members (UbE2E2 and UbE2E3), which share high sequence similarity to UbE2E1 and have redundant roles to UbE2E1 in many physiological processes. Further research is required to investigate whether UbE2E2 and UbE2E3 also contribute to regulation of H2A monoubiquitination.

Because it is well established that uH2AK119 is one of the epigenetic signatures for transcriptional repression of the *INK4A*/*ARF* locus, we used this locus to analyze the potential role of UbE2E1 in PRC1-dependent transcriptional regulation. We examined the levels of $p16^{INK4a}$ mRNA and protein, uH2AK119 occupancy of the *INK4A*/*ARF* locus, and cell growth by altering cellular UbE2E1 activity. We showed that UbE2E1 contributes to the transcriptional repression of *INK4A*/*ARF* by regulating monoubiquitination of H2A Lys-119. In all cases, the results are in agreement with the critical function of UbE2E1 in monoubiquitination of H2A Lys-119.

The role of USP7 in Polycomb group protein-mediated silencing has been reported previously. USP7 was shown to interact with guanosine monophosphate synthase and contribute to H2B deubiquitination and gene silencing in both humans and *Drosophila* (34, 35). In addition, USP7 and USP11 were co-purified with PRC1 complex proteins and shown to regulate the levels of $p16^{INK4a}$ (18). In this study, we further confirmed that USP7 plays an important role in maintaining the steady state level of PRC1 proteins especially UbE2E1 and Ring1B. In contrast to the study by Maertens *et al.* (18), we are able to readily and reproducibly detect a robust decrease in the level of uH2AK119 with USP7 knockdown or deletion, suggesting that USP7 also plays a role in regulating the level of uH2AK119. This is supported by Lecona *et al.* (21) who also observed decreased levels of uH2A in USP7 knock-out cells. We observed increased senescence and growth arrest in HCT116 $USP7^{-/-}$ cells, which can be partially explained by decreased uH2AK119 and increased $p16^{INK4a}$ levels in these cells.

Our study sheds new light into the regulation and function of the PRC1 complex. We identified UbE2E1 as a critical *in vivo* E2 for the PRC1-catalyzed monoubiquitination of H2A Lys-119. We also provided more evidence supporting USP7 as key regulator of the PRC1 complex by modulating PRC1 stability, activity, and function.

Experimental Procedures

Cell Culture and Antibodies—Human U2OS and HCT116 cells were grown in McCoy's media. HeLa cells were grown in DMEM supplemented with 10% FBS. HCT116 $USP7^{+/+}$ and $USP7^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (John Hopkins). The antibodies used for immunoblotting and immunostaining experiments include the mouse antibody to ubiquitin (clone P4G7, MMS-258R, Covance), actin (SC-1616, Santa Cruz), Myc (clone 4A6, 05–724, Millipore), FLAG M2 (F3165, Sigma), mouse, rabbit, and goat antibodies to UbE2E1 (611218, BD, A-630, Boston Biochem and SC-475478, Santa Cruz), USP7 (A300-033, Bethyl), Histone H2A (07–146, Millipore), ubiquitin-Histone H2A Lys-119 (clone D27C4, 8240, Cell Signaling), p16 (Neomarkers), UbcH5/UbE2D (A-615, Boston Biochem), Ring1A (clone D2P4D, 13069, Cell Signaling), CBX2 (ab80044, ABCAM), BMI1 (5856, Cell Signaling), Ring1B (clone D22F2, 5694, Cell Signaling and Active Motif), and H3K4me2/3 (ab6000, ABCAM). These antibodies have previously been used in our and other studies (11, 36–40).

Affinity Capture Proteomic Studies of UbE2E1—FLAG-tagged UbE2E1-expressing cells were harvested and resuspended in 4× (w:v) lysis buffer (50 mM HEPES (pH 8.0), 100 mM KCl, 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 1 mM DTT, and 1:500 protease inhibitor mixture (Sigma)). Resuspended cells were incubated with pre-equilibrated FLAG-M2-agarose beads (Sigma) for 2 h at 4 °C. Beads were pelleted by centrifugation at 1000 × *g* for 1 min, and then washed once with 1 ml of lysis buffer, and two times with 1 ml of ammonium bicarbonate rinsing buffer (50 mM ammonium bicarbonate (pH 8.0), 75 mM KCl). Elution was performed by incubation with 150 μl of 125 mM ammonium hydroxide (pH 11.0) and repeated twice more. 1 μg of mass spectrometry grade L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Promega) dissolved in 70 μl of 50 mM ammonium bicarbonate (pH 8.3) was added to the eluate and incubated at 37 °C overnight. The resulting peptides were lyophilized and resuspended in buffer A (0.1% formic acid). Liquid chromatography (LC) analytical columns (75 μm inner diameter) and pre-columns (100 μm) were prepared in-house from fused silica capillary tubing from InnovaQuartz and packed with 100Å C_{18}-coated silica particles (Magic, Michrom Bioresources). Peptides were subjected to nanoflow LC-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS), using a 120-min reversed phase LC (RPLC; 95% water-95% acetonitrile, 0.1% formic acid) buffer gradient running at 250 nl/min on a Proxeon EASY-nLC pump in-line with a hybrid LTQ-Orbitrap velos mass spectrometer (Thermo Fisher Scientific). A parent

ion scan was performed in the Orbitrap using a resolving power of 60,000, then up to 40 of the most intense peaks were selected for MS/MS (minimum ion count of 1000 for activation), using standard CID fragmentation. Fragment ions were detected in the LTQ. Dynamic exclusion was activated such that MS/MS of the same *m/z* (within a −0.1 and +2.1 Th window; exclusion list size = 500) detected 3 times within 45 s were excluded from analysis for 60 s. For protein identification, Thermo.RAW files were converted to .mzXML format using Proteowizard (41), then searched using X!Tandem (42) against the human RefSeq (Version 37) database. X!Tandem search parameters were: complete modifications, none; cysteine modifications, none; potential modifications, +16@M and W, +32@M and W, +42@N terminus, +1@N and Q. Data were analyzed using the ProHits (43) and SAINT software tools (44).

Immunoprecipitation—Co-immunoprecipitations were performed using FLAG-tagged UbE2E1 and endogenous Ring1A, as well as endogenous UbE2E1 and Ring1B. Cells were harvested 48 h after transfection and lysed in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate and protease inhibitor mixture (Roche Applied Science)). Lysates were incubated with antibodies against FLAG for FLAG-tagged UbE2E1 or Ring1B overnight at 4 °C followed by the addition of protein A beads for another 60 min. Immunoprecipitates were washed three times using RIPA buffer. The IP complexes were released by boiling the beads for 5 min in SDS sample buffer and resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies against UbE2E1, Ring1A, or Ring1B.

FLAG Pulldown Assay—Cells were transfected with FLAG-tagged Ring1B and BMI1. The cells were harvested after 48 h and lysed in buffer containing 50 mM Tris (pH 8.0), 500 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate and protease inhibitor mixture (Roche Applied Science). The cell lysate was incubated with FLAG-agarose to capture the FLAG-tagged Ring1B•BMI1 complex. After extensive washing, 1 mg of UbE2E1 or GST as negative control were incubated with the FLAG-tagged Ring1B•BMI1-immobilized agarose beads. The captured proteins were eluted with 3× FLAG peptide and resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies against Ring1B, BMI1, UbE2E1, and GST.

In Vitro Ubiquitination Assays—Ubiquitination of H2A was performed in a volume of 20 μl by incubating E1 (100 ng), E2 (UbE2E1 or UbE2D2, 200 ng), ubiquitin (5 μg), FLAG-tagged wild-type or mutant Ring1B•BMI1 complex, and 0.5 μg of reconstituted nucleosome in 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP, and 2 mM DTT at 30 °C for 90 min. The reaction was stopped by the addition of 5 μl of SDS-PAGE sample buffer and separated by 15% SDS-polyacrylamide gels. Ubiquitinated proteins were visualized and evaluated by Western blotting using specific antibodies against Ring1B, H2A, and uH2AK119. The proteins used in this assay were prepared as follows. E1 was expressed as His_6-tagged fusion protein using baculovirus-infected insect cell systems. E2s and ubiquitin were expressed as His_6-tagged fusion protein in *Escherichia coli*. E1, E2s, and ubiquitin were purified using affinity chromatography and prepared for assay as described previously (27). Recombinant nucleosome was prepared as previously described (46). The protein concentrations were estimated using the Bradford protein assay (Bio-Rad).

Overexpression of UbE2E1 and UbE2E1_C131A—Cells were transfected with UbE2E1 and UbE2E1_C131A. The cells were harvested 48 h after transfection and lysed. The lysate was resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies against uH2A, H2A, and methylated H3K4.

siRNA Knockdown of UbE2E1, UbE2Ds, or USP7—Cells were transfected with siRNAs for UbE2E1 (5′-GACCAAGAGA-UACGCUACA and 5′-GUGUAUUCUUUCUCGAUAU-3′, Genepharma), with siRNAs for UbE2Ds (5′-CAGUAAUG-GCAGCAUUUGUTT-3′ and 5′-GAUCACAGUGGUCGC-CUGCTT-3′, Genepharma), with siRNA for USP7 (5′-CCCAAAUUAUUCCGCGGCAAA-3′, Genepharma) or a negative control siRNA (provided by Genepharma). siRNA was transfected into cells using Lipojet (SignaGen) according to the manufacturer's protocol and cells were harvested 72 h post-transfection. The lysate was resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies as indicated.

Histone Extraction—The cells were lysed in 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF, 1 mM *N*-ethylmaleimide (NEM), and 10 μM ubiquitin aldehyde followed by the addition of hydrochloric acid (0.2 M final concentration) and incubated on ice for 30 min (34). The lysate was centrifuged and the soluble fraction was dialyzed against 0.1 M acetic acid followed by distilled water. The purified histones were analyzed by immunoblotting with antibodies against uH2AK119, H3K4me2/3, and H2A.

$p16^{INK4A}$ mRNA Expression Analysis—Total cellular RNA was extracted with TRIzol reagent (Invitrogen). RT-PCR was performed with 2 μg of total RNA using the ThermoScript™ RT-PCR kit (Invitrogen). The levels of $p16^{INK4A}$ mRNA and GAPDH mRNA were detected by quantitative PCR using the following primer pairs: 5′-GACCCCGCCACTCTCACC-3′ and 5′-CCTGTAGGACCTTCGGTGACTGA-3′ for $p16^{INK4A}$, and 5′-AAGGTCATCCCTGAGCTGAAC-3′ and 5′-ACGC-CTGCTTCACCACCTTCT-3′ for GAPDH. The $p16^{INK4A}$ expression levels were normalized to that of GAPDH.

Growth Curves and Senescence-associated β-Galactosidase Assay—Cell growth was measured by seeding equal numbers of each cell line or experimental condition in triplicate for each day of the experiment, followed by daily harvest and counting using a Coulter counter. The total numbers of cells were plotted *versus* day to generate a growth curve. Cells were prepared for β-galactosidase staining by fixation with PBS formaldehyde/glutaraldehyde equilibrated to pH 6 and stained for β-galactosidase activity as described (47).

Chromatin Immunoprecipitation and Quantitative PCR—Cells were either treated with UbE2E1 siRNA or its control, and harvested 72 h after transfection. Nuclear pellets were generated with 0.2% Triton X-100 and treated with 20 mM *N*-ethylmaleimide to block endogenous deubiquitinase activity. The pellets were washed twice and resuspended in cutting buffer (10 mM Tris (pH7.5), 60 mM KCl, 15 mM NaCl and 3 mM $CaCl_2$), followed by a 5-min treatment with monococcal nuclease (Worthington) stopped by the addition of EDTA. The supernatant was collected (S1) and the nuclear pellet was further incubated to release the poynucleosomal fraction (S2). The DNA

concentration was determined using 260/280 nm of the S2 fraction and used to allow equal inputs in the ChIP. S2 extracts were incubated with anti-uH2A or rabbit IgG overnight, followed by precipitation with protein A beads and washed with PBS containing 0.5% Nonidet P-40. The IP complex was dissociated with 1% SDS and DNA was precipitated using a phenol/chloroform extraction with glycogen as a carrier. Equal amounts of DNA (8 μg) from each ChIP fraction or input DNA (DNA extracted from the S2 fraction) were used as template in quantitative PCR using So fast master mix (Bio-Rad) and the following primer sets: Primer set 1, 4.5 kb downstream of *ARF* promoter (GGAGCGATGTGATCCGTTATC and TGAAATCCCAATCGTCTTCCAC); Primer set 2, 1 kb upstream of *INK4A* promoter (CTCAAAGCGGATAATTCAAGAGC and AAGCCTTAAGAACAGTGCCACAC); Primer set 3, Exon 2 of *INK4A* (CAAGCTTCCTTTCCGTCATGC and GCCAGAGAGAACAGAATGGTCAGAGCCA); and Primer set 4, 1.5 kb downstream of exon 3 (TGTCTACCCAACACTTCCCTGC and AAGGCAAAGGTAACTGAAACGC) (45).

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