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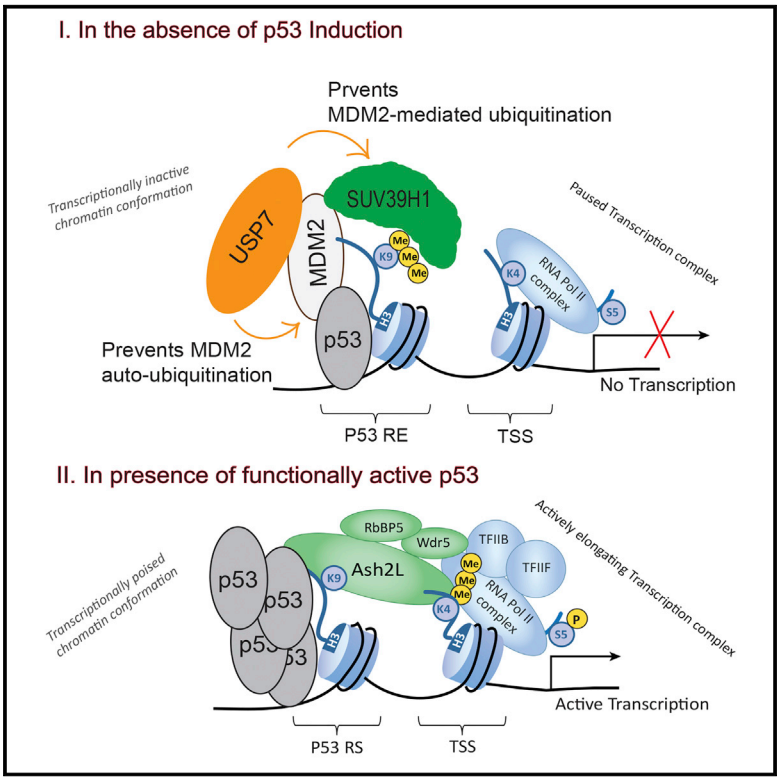
Report

USP7 Enforces Heterochromatinization of p53 Target Promoters by Protecting SUV39H1 from MDM2-Mediated Degradation

Graphical Abstract



Highlights

- USP7 enhances SUV39H1 stability
- USP7 forms a trimeric protein complex with SUV39H1 and MDM2
- USP7 occupies p53 target promoters and maintains the H3K9me3 mark
- Reduced USP7 activity enhances chemotherapy-induced p53-dependent apoptosis

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In Brief

Mungamuri et al. show that in the absence of a p53 stimulus, USP7 plays a role in maintaining the H3K9me3 repressive mark on p53 targets by protecting SUV39H1 from MDM2-mediated degradation and enforcing heterochromatinization of these promoters.

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Report

USP7 Enforces Heterochromatinization of p53 Target Promoters by Protecting SUV39H1 from MDM2-Mediated Degradation

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SUMMARY

The H3K9me3 repressive histone conformation of p53 target promoters is abrogated in response to p53 activation by MDM2-mediated SUV39H1 degradation. Here, we present evidence that the USP7 deubiquitinase protects SUV39H1 from MDM2-mediated ubiquitination in the absence of p53 stimulus. USP7 occupies p53 target promoters in unstressed conditions, a process that is abrogated with p53 activation associated with loss of the H3K9me3 mark on these same promoters. Mechanistically, USP7 forms a trimeric complex with MDM2 and SUV39H1, independent of DNA, and modulates MDM2-dependent SUV39H1 ubiquitination. Furthermore, we show that this protective function of USP7 on SUV39H1 is independent of p53. Finally, USP7 blocking cooperates with p53 in inducing apoptosis by enhancing p53 promoter occupancy and dependent transactivation of target genes. These results uncover a layer of the p53 transcriptional program mediated by USP7, which restrains relaxation of local chromatin conformation at p53 target promoters.

INTRODUCTION

TP53 is a well-established tumor suppressor and cellular gatekeeper of genome stability (Bieging et al., 2014; Khoo et al., 2014). In response to homeostatic stresses, p53 is stabilized and recruits core transcriptional machinery proteins to its target promoters, enabling transactivation of these genes, with cellular outcomes including cell-cycle arrest and apoptosis (Kruiswijk et al., 2015; Mandinova and Lee, 2011; Vousden and Prives, 2009; Zilfou and Lowe, 2009).

Chromatin conformation plays a major role in p53-dependent transcription (Allen et al., 2014; Beckerman and Prives, 2010; Botcheva, 2014; Su et al., 2015). p53 interacts with several cofactors, which have intrinsic histone-modifying activities (Liu et al., 1999; Vaziri et al., 2001), and with histone deacetylase complexes that act specifically to remodel chromatin (Brooks and Gu, 2011; Dai and Gu, 2010). We and others have shown that p53 target promoters are enriched with the H3K9me3 (histone H3 lysine9 trimethylation) mark and that p53 activation abrogates this repressive chromatin conformation through MDM2 proto-oncogene (MDM2)-mediated degradation of suppressor of variegation 3-9 homolog 1 (*Drosophila*) (SUV39H1) (KMT1A), a major methyltransferase responsible for writing this mark (Bosch-Presegué et al., 2011; Choi et al., 2012; Mungamuri et al., 2012; Zheng et al., 2014). Further, lysine 87 of SUV39H1 has been identified as the primary site of MDM2-mediated ubiquitination (Bosch-Presegué et al., 2011).

In the absence of genotoxic stress, p53 is known to occupy target promoters such as p21, Gadd45α, and PUMA but remains transcriptionally inactive (Allen et al., 2014; Espinosa et al., 2003; Jackson and Pereira-Smith, 2006; Kaeser and Iggo, 2002). Specifically, MDM2 has been proposed to repress p53 activity when co-occupied on these promoters, with p53 stress stimuli relieving such repression (Kruse and Gu, 2009; Minsky and Oren, 2004). There is also evidence that degradation of SUV39H1, also present on these same promoters, is required for p53-dependent transcription (Choi et al., 2012; Mungamuri et al., 2012). However, the mechanisms responsible for protecting SUV39H1 from MDM2-mediated degradation and preserving the repressive H3K9me3 mark in the absence of stress-induced p53 stabilization remain to be elucidated.

USP7 (ubiquitin-specific peptidase 7) is known to have a dual role in the p53-MDM2 pathway, as it deubiquitinates both p53 and MDM2. USP7 overexpression counteracts MDM2-mediated p53 ubiquitination, which stabilizes p53, leading to apoptosis (Li et al., 2004; Li et al., 2002). Disruption of the USP7 gene is also lethal to p53 wild-type (WT) cells, as loss of USP7 expression enhances the auto-ubiquitination of MDM2 leading to its degradation, resulting in p53 stabilization and apoptosis (Cummins et al., 2004; Cummins and Vogelstein, 2004). The present studies define a role of USP7 in the regulation of MDM2-mediated SUV39H1 degradation and maintenance of the H3K9me3 repressive chromatin conformation on p53 target promoters in unstressed cells.

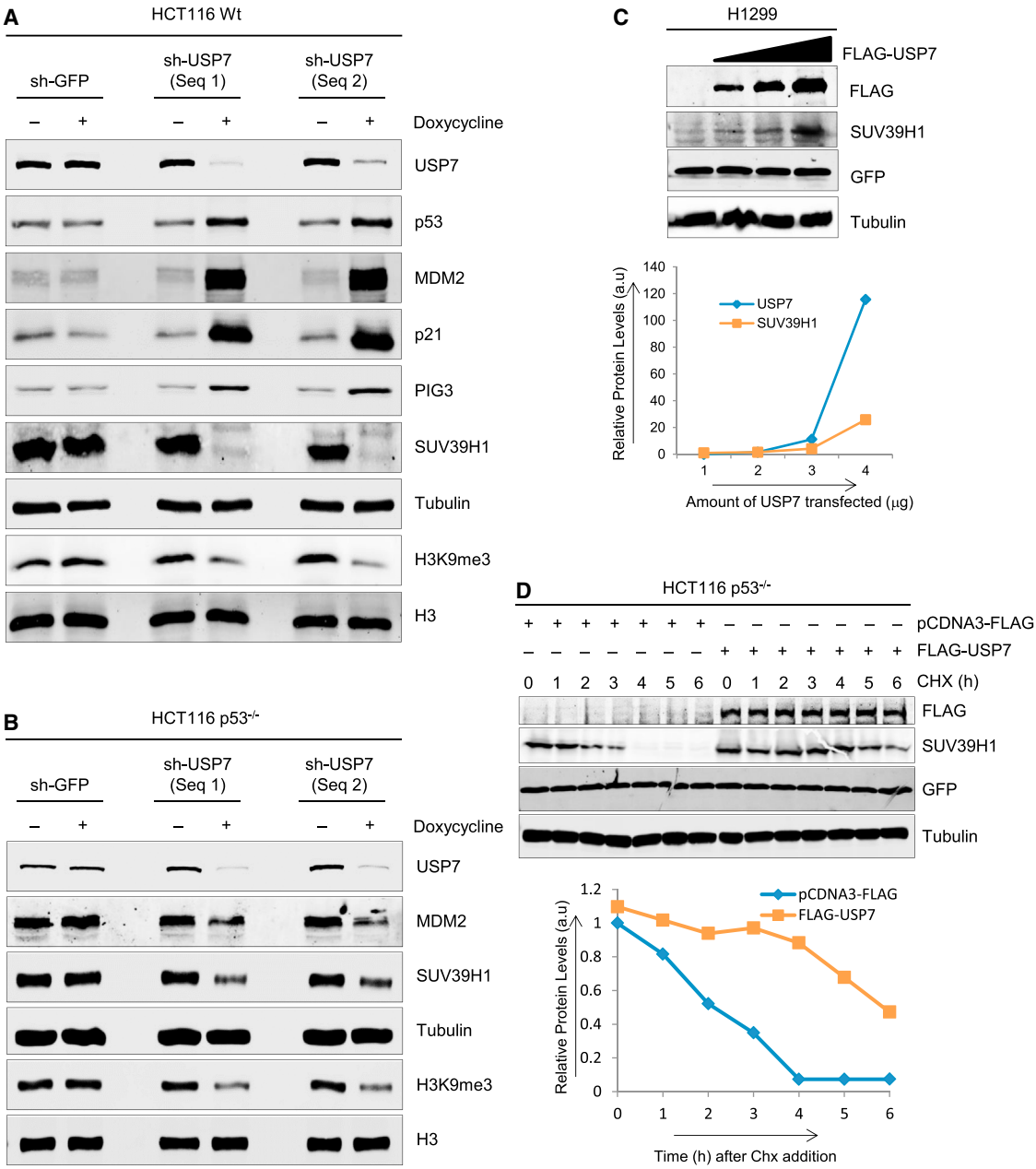

Figure 1. USP7 Enhances SUV39H1 Protein Stability Independent of p53

(A and B) Western blot analysis of HCT116 p53 WT (A) and HCT116 p53$^{-/-}$ (B) cells stably transduced with doxycycline-inducible shGFP or shUSP7 (two sequences) and cultured in the presence of doxycycline for 48 hr.

(C) Western blot analysis of H1299 cells transiently overexpressing 1, 2, or 4 μg FLAG-USP7 for 24 hr. The amounts of proteins expressed (arbitrary units) are shown as a line diagram.

(D) Western blot analysis of HCT116 p53$^{-/-}$ cells transiently overexpressing FLAG-USP7 and treated with cycloheximide for indicated time points. The amounts of proteins expressed (arbitrary units) are shown as a line diagram.

See also Figures S1 and S2.

RESULTS

USP7 Enhances SUV39H1 Stability Independent of p53

To investigate whether USP7 modulates SUV39H1 expression levels, we generated HCT116 p53 WT cells stably expressing doxycycline-inducible USP7 small hairpin RNA (shRNA). The addition of doxycycline to the culture medium reduced USP7 expression as measured at both the mRNA (Figure S1A) and protein (Figure 1A) levels, and as expected, USP7 silencing stabilized p53, resulting in induction of its target genes (Figures 1A

and S1A). We showed previously that p53 regulates SUV39H1 at the mRNA level through p21 and at the protein level through MDM2 (Mungamuri et al., 2012). Consistent with these data, we observed a decrease in SUV39H1 mRNA and protein levels under these conditions (Figures 1A and S1A), which also correlated with loss of the H3K9me3 histone mark (Figure 1A). As a control, we generated similar stables in isogenic HCT116 $p53^{-/-}$ cells and analyzed the effect of USP7 silencing on SUV39H1 levels. USP7 knockdown in these cells had no effect on either the induction of p21 or downregulation of SUV39H1 mRNA levels (Figure S1B), but it resulted in reduction both of SUV39H1 protein expression and H3K9me3 mark levels (Figure 1B). Further, downregulation of SUV39H1 protein expression in response to USP7 silencing was inhibited when cells were treated with the proteasomal inhibitor MG-132 (Figure S1C). All of these findings indicated that USP7 loss destabilizes the SUV39H1 protein independent of p53.

To further investigate the role of USP7 in regulating SUV39H1 protein stability, we exogenously overexpressed USP7 in p53 null H1299 cells and observed a USP7 dose-dependent increase in SUV39H1 protein levels (Figure 1C). These results further substantiated that USP7 modulates steady-state SUV39H1 levels independent of p53. Finally, we transiently overexpressed USP7 in HCT116 $p53^{-/-}$ cells, treated with cycloheximide to block new protein synthesis, and observed a significant increase in SUV39H1 half-life (Figure 1D), confirming that USP7 positively regulates SUV39H1 protein stability.

USP7 Protects SUV39H1 from MDM2-Mediated Degradation

MDM2 ubiquitinates SUV39H1 and targets it for proteasomal degradation (Bosch-Presegué et al., 2011; Mungamuri et al., 2012). Thus, we tested whether USP7 protects SUV39H1 from MDM2-mediated degradation. Transient transfection of H1299 or HCT116 $p53^{-/-}$ cells revealed that exogenous MDM2 expression led to downregulation of exogenous SUV39H1 protein levels (Figures 2A and S2A), while USP7 co-transfection rescued SUV39H1 expression in a dose-dependent manner (Figure 2A). Exogenous MDM2 expression in HCT116 $p53^{-/-}$ cells downregulated levels of exogenously expressed SUV39H1 WT, but not an MDM2-resistant SUV39H1-K87A mutant, confirming previous findings (Bosch-Presegué et al., 2011) that lysine 87 is the site of ubiquitination by MDM2 (Figure S2A). Finally, doxycycline-induced shUSP7 expression in HCT116 $p53^{-/-}$ cells resulted in downregulation of exogenously expressed SUV39H1 WT, but not SUV39H1-K87A, protein levels (Figure S2A). These results support the conclusion that endogenous USP7 protects SUV39H1 from endogenous MDM2-mediated degradation.

USP7 activity can also be inhibited at sub-micromolar concentrations by HBX41108, a cyano-indenopyrazine derivative (Colland et al., 2009). In MDM2 non-silenced p53-null H1299 cells, USP7 inhibition using HBX41108 resulted in reduced levels of SUV39H1 WT, but not SUV39H1 K87A (site of MDM2 ubiquitination) (Figure S2B). When the same cells were pre-silenced for MDM2, HBX41108 treatment did not result in a decrease in either SUV39H1 WT or SUV39H1 K87A protein levels (Figure S2B). These results indicate that MDM2 is the E3 ligase for SUV39H1 (ubiquitinating at K87 residue) and that USP7 acts to stabilize SUV39H1 only in the presence of basal levels of MDM2. Furthermore, a USP7 catalytically inactive mutant (C223S), as well as the WT protein's N-terminal domain (NTD) or C-terminal domain (CTD), were unable to protect SUV39H1 from MDM2-mediated degradation, substantiating that functional, full-length USP7 is required for this protection (Figure 2B). In previous studies, we observed that MDM2-mediated SUV39H1 loss correlated with global reduction of the H3K9me3 mark (Mungamuri et al., 2012). In good agreement, USP7-mediated protection of SUV39H1 from MDM2-mediated degradation rescued H3K9me3 levels in the same cells, under the same conditions (Figures 2A, 2B, and S2A).

Overexpressed SUV39H1 displayed constitutive ubiquitination, which was enhanced by exogenous MDM2 expression (Figure 2C), whereas exogenous USP7 expression decreased this ubiquitination in a dose-dependent manner (Figure 2C). Collectively, these findings argue strongly that USP7 protects SUV39H1 from MDM2-mediated degradation.

USP7 Forms a Trimeric Protein Complex with SUV39H1 and MDM2

USP7 has been shown to protect both MDM2 from auto-ubiquitination and p53 from MDM2-mediated ubiquitination via physical interactions (Cummins et al., 2004; Li et al., 2002; Sheng et al., 2006). Thus, we tested the nature of the interactions, if any, among USP7, SUV39H1, and MDM2. Immunoprecipitation followed by immunoblot analysis of endogenous proteins using USP7, SUV39H1, or MDM2 antibody in HCT116 WT cells resulted in pull-down of the other two endogenous proteins, implying the presence of a tri-molecular protein complex in unstressed cells (Figure 2D). Further, this tri-molecular complex was equally detectable in cell lysates treated with ethidium bromide (Figure 2E). Whether or not the chromatin landscape, specifically the p53 RE (response element), is required for formation of this tri-molecular complex, these findings suggest that its stability is independent of DNA.

To further understand the role of USP7 catalytic activity and protein domains required for its interaction with SUV39H1, we used various tagged USP7 constructs. Co-expression of FLAG-tagged USP7 WT and SUV39H1, followed by FLAG-antibody pull-down and immunoblot analysis, established that USP7 forms an easily detectable protein complex with SUV39H1 (Figure S3A). Furthermore, a myc-tagged USP7-C223S mutant was also able to pull down SUV39H1 to a similar extent as myc-tagged USP7 WT (Figure S3B). These results indicated that USP7-SUV39H1 complex formation was independent of USP7 catalytic activity. Next, we asked which domains of USP7 mediate this interaction by co-immunoprecipitation experiments using myc-tagged constructs expressing different USP7 domains. While the USP7-NTD was able to pull down SUV39H1 to a similar extent as full-length USP7, the USP7-CTD showed no detectable interaction (Figure S3B), indicating that the NTD of USP7 is essential for protein complex formation with SUV39H1.

Previous studies have shown that MDM2 physically interacts with USP7 (Sheng et al., 2006) and also forms a complex with SUV39H1 (Fåhraeus and Olivares-Illana, 2014). We observed a trimolecular protein complex among endogenous USP7, SUV39H1, and MDM2 (Figures 2D and 2E). To assess whether

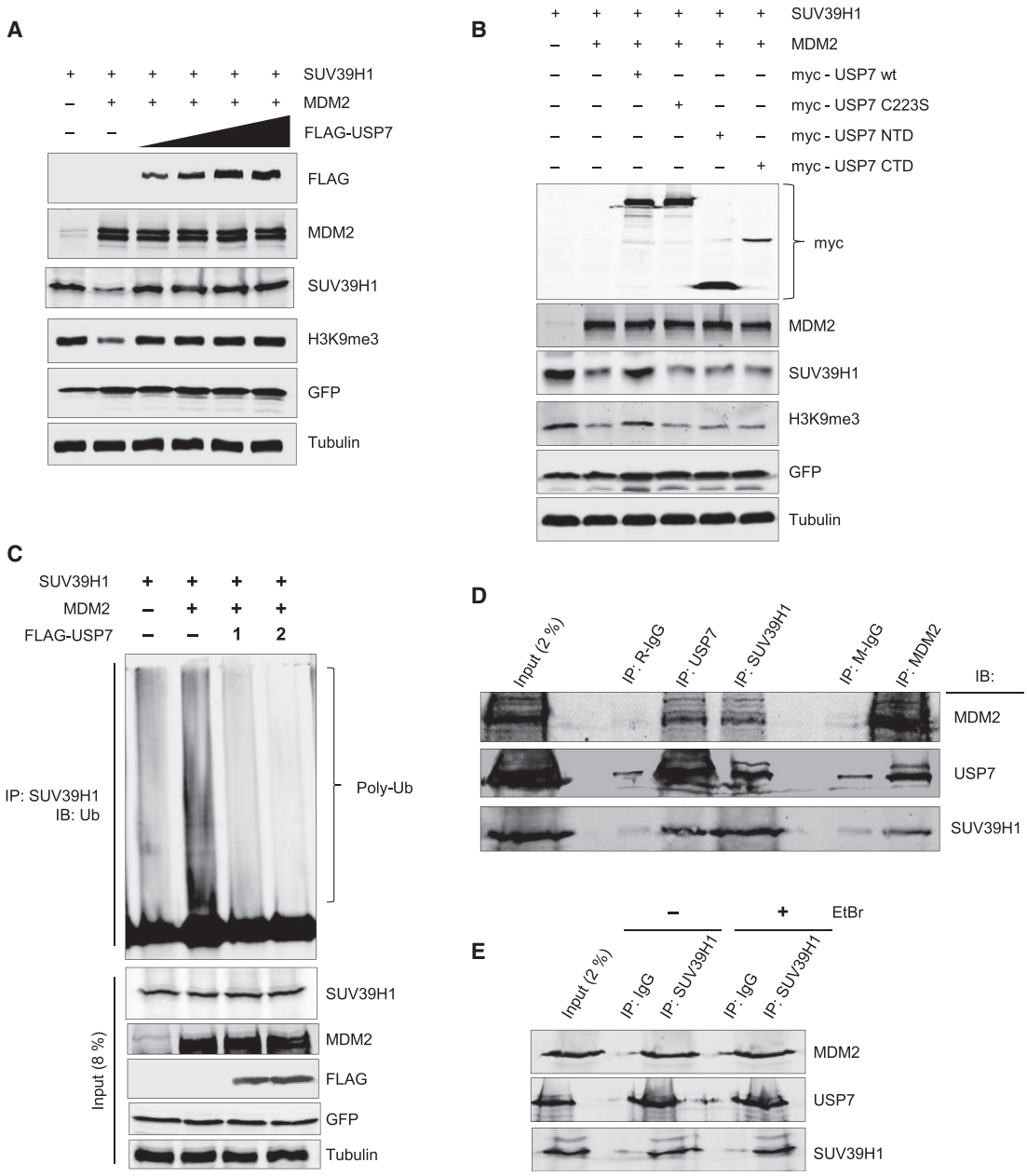

Figure 2. USP7 Interacts with SUV39H1 through MDM2 and Protects SUV39H1 from MDM2-Mediated Degradation

(A) Western blot analysis of H1299 cells transfected with SUV39H1, MDM2 and 1, 2, 3, or 4 μg FLAG-USP7 for 24 hr.

(B) Western blot analysis of H1299 cells transfected with SUV39H1, MDM2 and myc-tagged USP7 WT, C223S, NTD, or CTD expression constructs for 24 hr.

(C) Western blot analysis of SUV39H1 immunoprecipitated samples. H1299 cells were transfected with the indicated plasmids and 24 hr later SUV39H1 protein was immunoprecipitated and loaded on a gradient SDS-PAGE gel and probed with an anti-ubiquitin antibody. 8% of the sample used for immunoprecipitation (IP) was also loaded and showed as the input.

(D) Western blot analysis of immunoprecipitates from HCT116 p53 WT cell lysates. 2% of the sample used for IP was also loaded and showed as the input.

(E) Western blot analysis of immunoprecipitates from HCT116 p53 WT cell lysates. The cell lysates for treated with EtBr to denature the DNA, before performing immunoprecipitation. 2% of the sample used for IP was also loaded and showed as the input.

See also Figure S3.

USP7-SUV39H1 complex formation was dependent on MDM2, we performed USP7 immunoprecipitation in the absence and presence of MDM2 silencing (Figure S3C). USP7 was unable to form a protein complex with SUV39H1 in MDM2 pre-silenced cells (Figure S3D). Moreover, MDM2 silencing in $p53^{-/-}$ cells did not increase SUV39H1 steady-state levels, arguing that USP7 stabilization of SUV39H1 occurred only in the presence of MDM2 (Figure S3E). Further, overexpression of USP7 enhanced

the endogenous study-state levels of SUV39H1 only in control cells, but not in the cells in which MDM2 was pre-silenced (Figure S3F). Collectively, these results indicate that shared physical interactions of USP7 and SUV39H1 with MDM2 are required either for USP7 to deubiquitinate SUV39H1 or for MDM2 to ubiquitinate SUV39H1, both resulting in enhanced SUV39H1 stability. Further, our data that silencing MDM2 in p53-null cells does not further stabilize SUV39H1 argue that silencing MDM2 not only abrogated the degradation of SUV39H1 but also inhibited SUV39H1 interaction with USP7 and thus as a net effect has no change in SUV39H1 protein levels.

USP7 Occupies p53 Target Promoters and Maintains the H3K9me3 Mark

One possible mechanism for retention of SUV39H1/ H3K9me3 mark on p53 target promoters in unstressed p53 WT cells could be that USP7 is also recruited at these sites and protects SUV39H1 from MDM2-mediated degradation. In fact, chromatin immunoprecipitation (ChIP) analysis indicated that USP7 was enriched on p53 target promoters in HCT116 (Figure S4A) and B5/589 cells (Figure S4B), both harboring WT p53.

In response to nutlin3a treatment, we observed increased p53 occupancy on its target promoters (Figure S4C), associated with a decrease in USP7 occupancy on these same p53 target promoters (Figures S4A and S4B). To further validate these data, we performed a time-course analysis (0, 1, 2, 3, and 4 hr) of nutlin3 treatment of p53 WT cells to investigate how p53 enrichment influences the presence of USP7 and the H3K9me3 mark on p53 target promoters. The results establish that as p53 promoter occupancy increases with time of nutlin3 treatment, there is a decrease in USP7 occupancy and H3K9me3 enrichment on these same promoters (Figures S5B–S5D). Moreover, these results establish that the loss of USP7 occupancy and H3K9me3 enrichment on p21 and PUMA promoters precede detectable increases in p53 promoter occupancy. All of these findings are consistent with a model in which basal recruitment of MDM2 by p53 leads to basal recruitment of USP7-SUV39H1 and a basal level of H3K9me3. Under these same conditions, we observed no detectable changes in total cellular USP7 levels, as measured at both mRNA (Figures S6A and S6B) and protein (Figures S6C and 5A) levels.

We showed previously that the H3K9me3 repressive histone mark is enriched on p53 target promoters (Mungamuri et al., 2012, 2014). USP7 knockdown by shRNA reduced total cellular USP7 levels (Figures 1A and 1B) as well as USP7 promoter occupancy in p53 WT cells (Figure 3A). Pharmacological inhibition of USP7 did not reduce the total cellular pool of USP7 either in p53 WT or in p53-null cells, as measured at both the mRNA (Figure S6D) and protein (Figure 3B) levels. In HCT116 p53 WT cells, such inhibition stabilized p53 protein (Figure 3B), associated with enhanced p53 promoter occupancy (data not shown) and transactivation of p53 target genes (Figures 3B and S6D), as well as reduced USP7 occupancy on these same p53 target promoters (Figure 3C). In contrast, in p53-null cells, HBX41108 treatment did not induce p53 pro-apoptotic target gene expression (Figure S6D). These results, collectively argue that p53 stabilization/promoter occupancy is required for removal of USP7 from p53 target promoters.

Both shRNA-mediated USP7 silencing and pharmacological USP7 inhibition in HCT116 p53 WT cells led to global reduction of H3K9me3 levels (Figures 1A and 3B) as well as abrogation of H3K9me3 mark enrichment on each of the p53 target promoters analyzed (Figures 3D and 3E). Further, the loss of USP7 occupancy on p53 target promoters correlated with the abrogation of the H3K9me3 mark on these same promoters in a time-dependent manner (Figure S5D). These results argue that functional USP7 is required to maintain SUV39H1-dependent H3K9me3 enrichment on p53 target promoters.

Reduced USP7 Expression/Activity Enhances Chemotherapy-Induced p53-Dependent Apoptosis

Next, we sought to determine the functional significance of USP7-mediated stabilization of SUV39H1 on p53-dependent cell fate decisions. Etoposide, a topoisomerase II inhibitor, induced apoptosis in HCT116 p53 WT cells in a dose-dependent manner, as analyzed by propidium iodide staining (Figures 4A and S7A). The same cells showed significantly increased apoptosis when either USP7 was pharmacologically inhibited by pre-treatment with HBX41108 (Figures 4A and S7A) or when USP7 expression was inhibited using shRNA (Figure S8). The increases in apoptosis observed at different levels of etoposide in combination with HBX41108 and with shUSP7 were greater in each case than the sum of either HBX41108 / shUSP7 or etoposide treatment alone. Further, this enhanced apoptosis correlated well with increased induction of p53 target genes as measured at both protein (Figure 4B) and mRNA levels (Figures 4C and S7B). HBX41108 also cooperated with nutlin3a-stablized p53 to transactivate its target genes (data not shown), which was associated with enhanced p53 promoter occupancy (Figure 4D). Further, we also analyzed the H3K9me3 enrichment on p53 target promoters in HCT116 p53 WT cells under these conditions. The results show that while etoposide or HBX41108 treatment alone decreased H3K9me3 enrichment on p53 target promoters, their combined treatment further downregulated H3K9me3 enrichment on these same promoters (Figure 4E). Thus, loss of USP7 function favors increased p53 promoter occupancy and dependent transcription of its target genes through abrogation of the H3K9me3 mark.

Finally, we confirmed the enhanced p53 occupancy/transactivation in response to USP7 inhibition was indeed due to abrogation of the H3K9me3 mark on its target promoters, using HCT116 p53 WT cells stably overexpressing either SUV39H1 WT or SUV39H1-K87A. In contrast to vector stables, expression of p53 targets was inhibited in response to etoposide or HBX41108-mediated stabilization of p53 in cells stably overexpressing SUV39H1 WT (Figure 4B). In SUV39H1-WT-overexpressing cells, etoposide and HBX41108, either as single agents or in combination, induced p53 targets less efficiently compared to vector stables and were associated with higher residual levels of SUV39H1 (Figure 4B). In SUV39H1-K87A-overexpressing cells, neither etoposide nor HBX41108 alone or in combination was able to induce p53 target genes (Figure 4B). In good agreement, we did not observe degradation of SUV39H1-K87A under any of these conditions (Figure 4B), despite comparable levels of p53 stabilization and MDM2 induction across all of the cells analyzed (Figure 4B). All of these findings argue that decreased

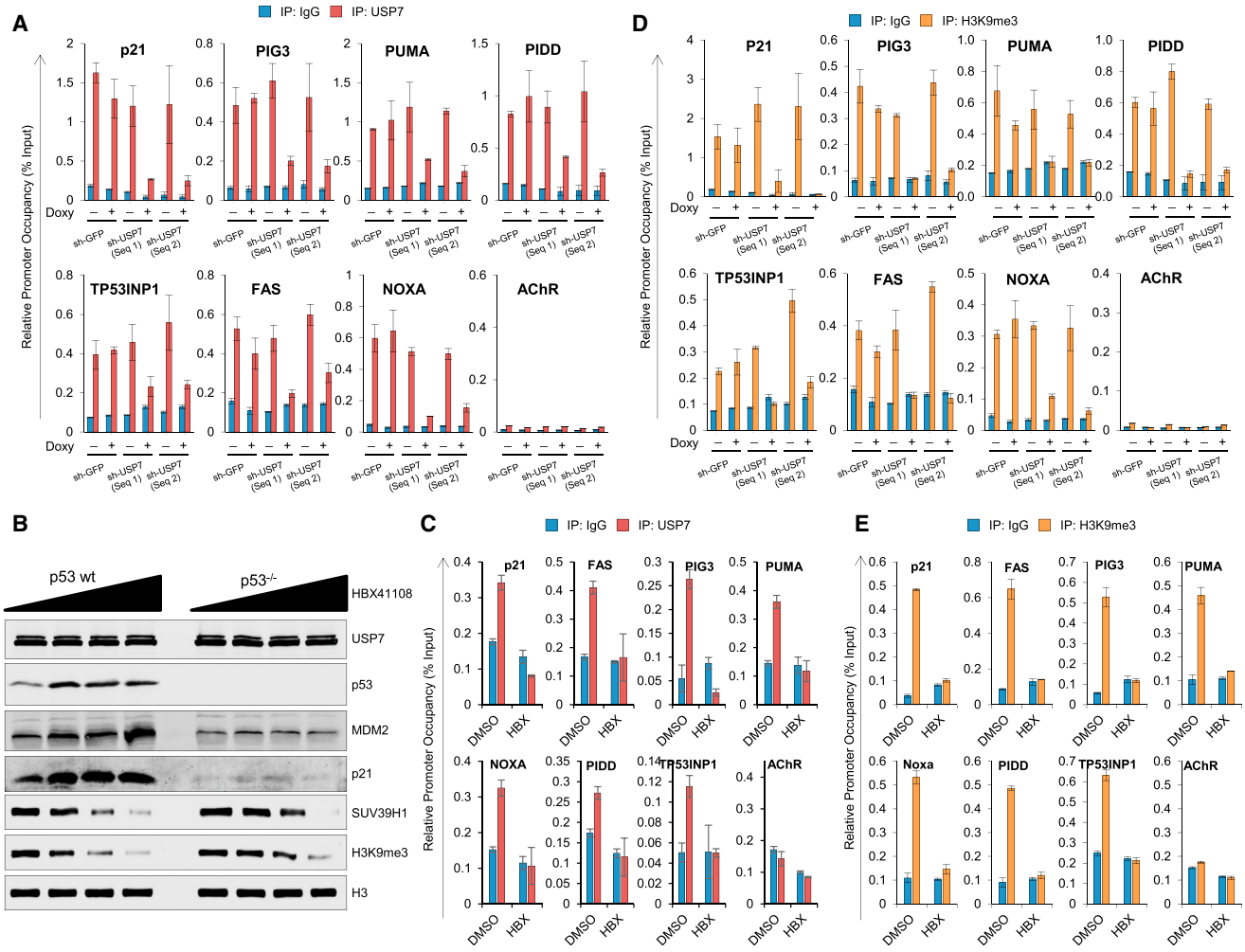

Figure 3. USP7 Occupies p53 Target Promoters and Maintains the H3K9me3 Mark on These Promoters

(A) ChIP analysis showing USP7 occupancy on p53 target promoters in HCT116 WT cells stably transduced with inducible shGFP or shUSP7 (two sequences) and cultured in the presence of doxycycline for 48 hr.

(B) Western blot analysis of HCT116 p53 WT and p53$^{-/-}$ cells treated with 0, 2.5, 5, or 10 μM HBX41108 for 24 hr.

(C) ChIP analysis showing H3K9me3 enrichment on p53 target promoters in HCT116 p53 WT cells stably transduced with inducible shGFP or shUSP7 (two sequences) and cultured in the presence of doxycycline for 48 hr.

(D and E) ChIP analysis showing USP7 occupancy (D) and H3K9me3 enrichment (E) in HCT116 p53 WT cells treated with 10 μM HBX41108 for 24 hr.

See also Figures S4–S6.

USP7 expression/activity leads to abrogation of the H3K9me3 repressive histone mark on p53 target promoters, enhancing p53-promoter occupancy, transactivation of its target genes, and the pro-apoptotic response.

SUV39H1 is known to influence global gene expression (Kondo et al., 2008; Peters et al., 2001), and we reported previously that p53 regulates the expression of growth hormone receptor (GHR), myostatin (GDF8), scinderin (SCIN), and *ets* homologous factor (EHF) through downregulation of SUV39H1, even though these gene promoters lack p53 binding sites (Mungamuri et al., 2012). Thus, we analyzed the expression of these same genes in response to USP7 and MDM2 silencing. While USP7 silencing induced the expression of GHR, GDF8, SCIN, and EHF (Figure S9A), silencing MDM2 had no effect on the induction of these genes (Figure S9B). All of these results argue that USP7 regulates SUV39H1 steady-state protein levels and that downregulation of USP7 mimics downregulation of SUV39H1, leading to abrogation of the H3K9me3 mark and transactivation of target promoters. Further studies will be necessary to determine the global effects on gene expression mediated by USP7 and specifically how many of these changes can be attributed to its modulation of SUV39H1/H3K9me3 levels.

DISCUSSION

A number of studies have indicated that in unstressed WT p53 harboring cells, p53 occupies target promoters yet remains transcriptionally inactive (Espinosa et al., 2003; Jackson and

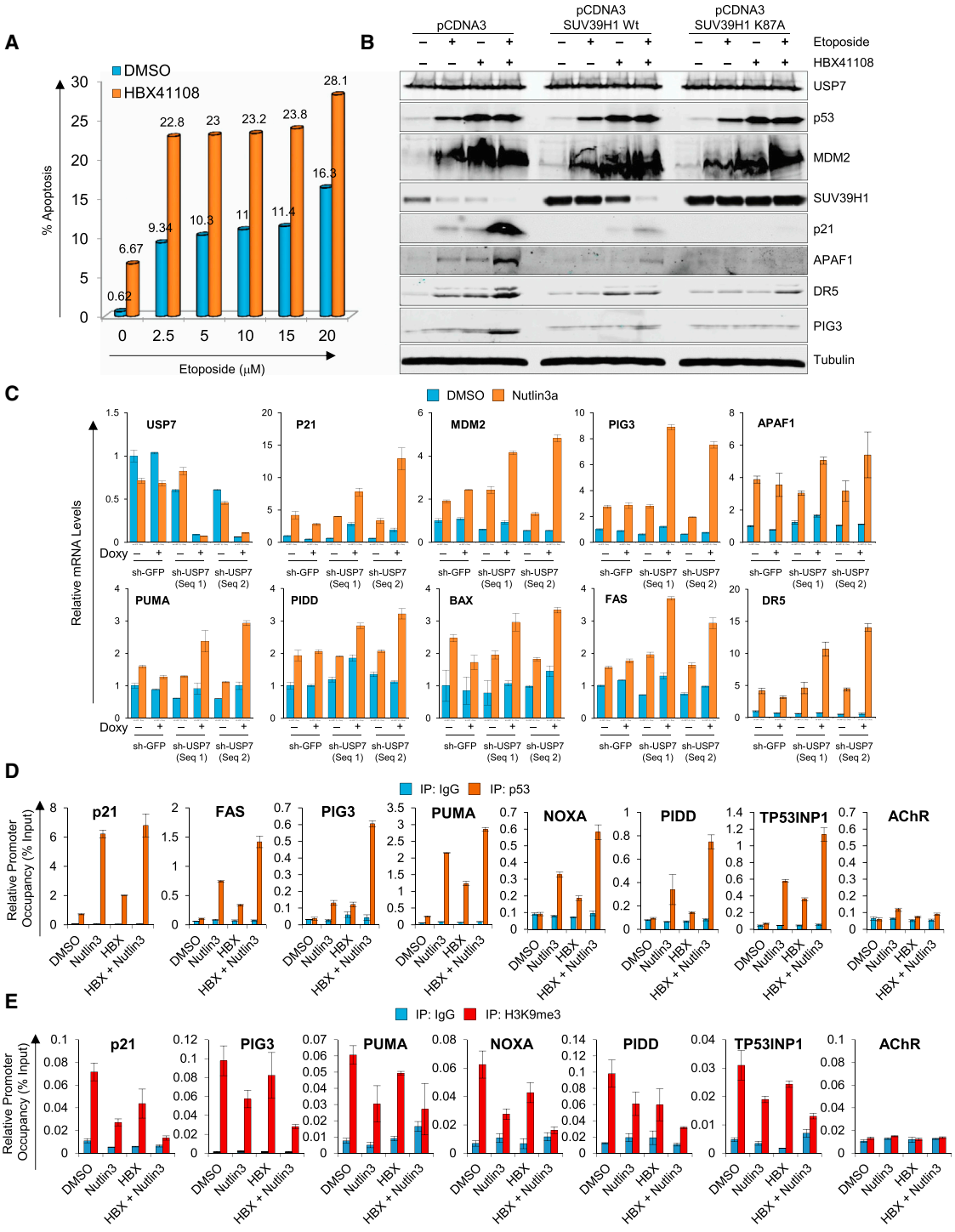

Figure 4. Pharmacological Inhibition of USP7 or USP7 Pre-silencing Enhances Chemotherapy-Induced Apoptosis in a p53-Dependent Manner

(A) Propidium iodide (PI) staining in HCT116 p53 WT cells pre-treated with either DMSO or 2.5 μM HBX41108 for 24 hr followed by treatment with increasing doses of etoposide for 48 hr. The percentage of cells showing less than a 2N content of DNA (apoptosis) in each condition is shown in the table (see Figure S7A for actual fluorescence-activated cell sorting [FACS] graphs).

(B) Western blot analysis of HCT116 p53 WT cells stably expressing SUV39H1 WT or SUV39H1-K87A are pre-treated with either DMSO or 2.5 μM HBX41108 for 24 hr followed by treatment with 5 μM of etoposide for another 24 hr.

(legend continued on next page)

Pereira-Smith, 2006; Kaeser and Iggo, 2002). An anti-repression mechanism has been postulated in which MDM2 co-occupies these promoters and represses p53-dependent transcription (Kruse and Gu, 2009; Minsky and Oren, 2004). Cellular stresses, which stabilize p53, result in MDM2 promoter clearance, thus relieving MDM2 repression and allowing p53 to function. The presence of the H3K9me3 histone repressive mark represents another major obstacle to p53 transactivation in unstressed cells. We recently established that the repressive chromatin conformation associated with this mark inhibits p53-dependent transcription of its target genes and that p53 overcomes such repression through MDM2-mediated degradation of SUV39H1, the writer of this mark (Mungamuri et al., 2012).

The MDM2 E3 ubiquitin ligase is both a p53 transcriptional target and in a negative feedback loop targets p53 for proteasomal degradation (Beckerman and Prives, 2010). The USP7 deubiquitinase is known to modulate the p53 pathway by protecting p53 from MDM2-mediated degradation (Cummins et al., 2004) and MDM2 from auto-ubiquitination (Li et al., 2004). Evidence that USP7 is present in the chromatin fraction (Maertens et al., 2010) and that MDM2 also degrades SUV39H1 led us to investigate a possible role of USP7 in protecting SUV39H1 from MDM2-mediated degradation. Our present studies establish that USP7 is enriched on p53 target promoters and modulates SUV39H1/H3K9me3 levels in an MDM2-dependent manner. Moreover, either USP7 knockdown or a USP7 small-molecule inhibitor led to a global decrease in SUV39H1 protein and H3K9me3 levels as well as abrogation of the H3K9me3 mark on p53 pro-apoptotic target promoters. Thus, our findings support the concept that USP7 through its interactions with SUV39H1 and MDM2 plays an important role in enforcing MDM2-mediated repression of p53-dependent transcription of its target genes in unstressed cells.

Previous reports have indicated that MDM2 binds USP7 through interactions mediated by MDM2 aa 147–159 (Sheng et al., 2006), while aa 200–262 are involved in MDM2 binding to SUV39H1 (Fåhraeus and Olivares-Illana, 2014). Our present studies demonstrate that USP7, SUV39H1, and MDM2 exist in a trimeric complex and that the USP7-MDM2-SUV39H1 complex keeps MDM2 inactive under basal conditions. These findings are consistent with a model in which USP7 regulates SUV39H1 protein levels indirectly by modulating MDM2 E3 ligase activity. Alternatively, MDM2 binding of both USP7 and SUV39H1 may place these molecules in close proximity such that low-affinity interaction not detectable in the absence of MDM2 may allow USP7 to directly deubiquitinate SUV39H1. By either mechanism, our results establish that USP7 modulates the repressive H3K9me3 mark by protecting SUV39H1 from MDM2-mediated degradation and blocking aberrant p53 transactivation of target genes in unstressed p53 WT cells. Whether this protection occurs on p53 target promoters alone and/or elsewhere within the cell as well as the physiological significance of these interactions in a p53 null context remain to be elucidated.

Our present findings as well as data available from previous reports indicate that "basal" levels of mostly transcriptionally inactive p53 enable the recruitment of the USP7-MDM2-SUV39H1 complex to p53 REs via p53-MDM2 interaction, placing the H3K9me3 repressive mark and maintaining low basal transcription activity of p53 target genes (Figure 5). Upon p53 activation by stress stimuli or nutlin3, p53-MDM2 interaction is disrupted, leading to increased p53 accumulation and chromatin binding, leading to productive transcription. MDM2 accumulation, as a result of p53-dependent transcription, blocks SUV39H1-mediated H3K9me3 enrichment and its repressive effect by triggering SUV39H1 degradation. Thus, p53-dependent MDM2 accumulation serves two purposes: (1) a negative feedback loop via p53 degradation once the stressful stimulus is relieved and (2) a fast-forward loop whereas p53 transactivation is enhanced via degradation of the SUV39H1.

Several studies have shown that USP7 directly affects chromatin structure by deubiquitinating and stabilizing histone H2A (Lecona et al., 2015) and histone acetyl transferase TIP60 (Gao et al., 2013), both of which enhance p53 function (Dar et al., 2013; Shema et al., 2008). Our present findings demonstrate that USP7 also has a major impact on p53 pro-apoptotic signaling by altering H3K9me3 enrichment on p53 target promoters, uncovering a new layer of p53 regulation at the chromatin level. We showed that inhibition of USP7 function through shRNA-mediated knockdown or by pharmacological inhibition cooperated with chemotherapy in inducing apoptosis in a p53-dependent manner by favoring p53 promoter occupancy and transactivation of its target genes. Such cooperation may be clinically beneficial in allowing the use of lower levels of chemotherapy to achieve a better therapeutic index (Cheon and Baek, 2006; Nicholson and Suresh Kumar, 2011).

EXPERIMENTAL PROCEDURES

Cell Lines, Plasmids, and Treatments

HCT116 p53 WT, HCT116 p53$^{-/-}$, B5/589 (p53 WT), and H1299 (p53 null) (Mungamuri et al., 2012; Mungamuri et al., 2014) cancer cells were used. Specific gene silencing was achieved by using either pTripZ (Open Biosystems) or Tet-pLKO-puro (#21915; Addgene) vectors. Human SUV39H1 cDNA is previously described (Mungamuri et al., 2012). MDM2 and FLAG-USP7 cDNA (Cummins et al., 2004; Zhou et al., 2001) were obtained from Addgene (#16233 and #16655). Myc-USP7 WT and other deletion constructs are described previously (Sarkari et al., 2010). Please see the Supplemental Experimental Procedures for the list of chemicals and their concentrations used in this study.

Real-Time qPCR and Western Blotting

RNA extraction and real-time qPCR was performed as described previously (Mungamuri et al., 2012). Western blotting and flow cytometry were done as described previously (Mungamuri et al., 2012). Please see the Supplemental Information for the list of antibodies used in the study. All blots were developed using the Odyssey fluorescence image scanner. Please see Table S1 for the sequences of primers used.

(C) Real-time qPCR analysis of HCT116 p53 WT cells stably transduced with inducible shGFP or shUSP7 (two sequences) and cultured in the presence of doxycycline for 24 hr (instead of 48 hr, in order to minimize the extent of p53 activation of its target genes and optimize the ability to detect cooperation) followed by treating cells with 10 μM nutlin3a for another 16 hr.

(D and E) ChIP analysis showing p53 occupancy (D) and H3K9me3 enrichment (E) on p53 target promoters in HCT116 p53 WT cells pretreated with either DMSO or 2.5 μM HBX41108 for 24 hr followed by treatment with 10 μM nutlin3a for another 16 hr. See also Figures S7–S9.

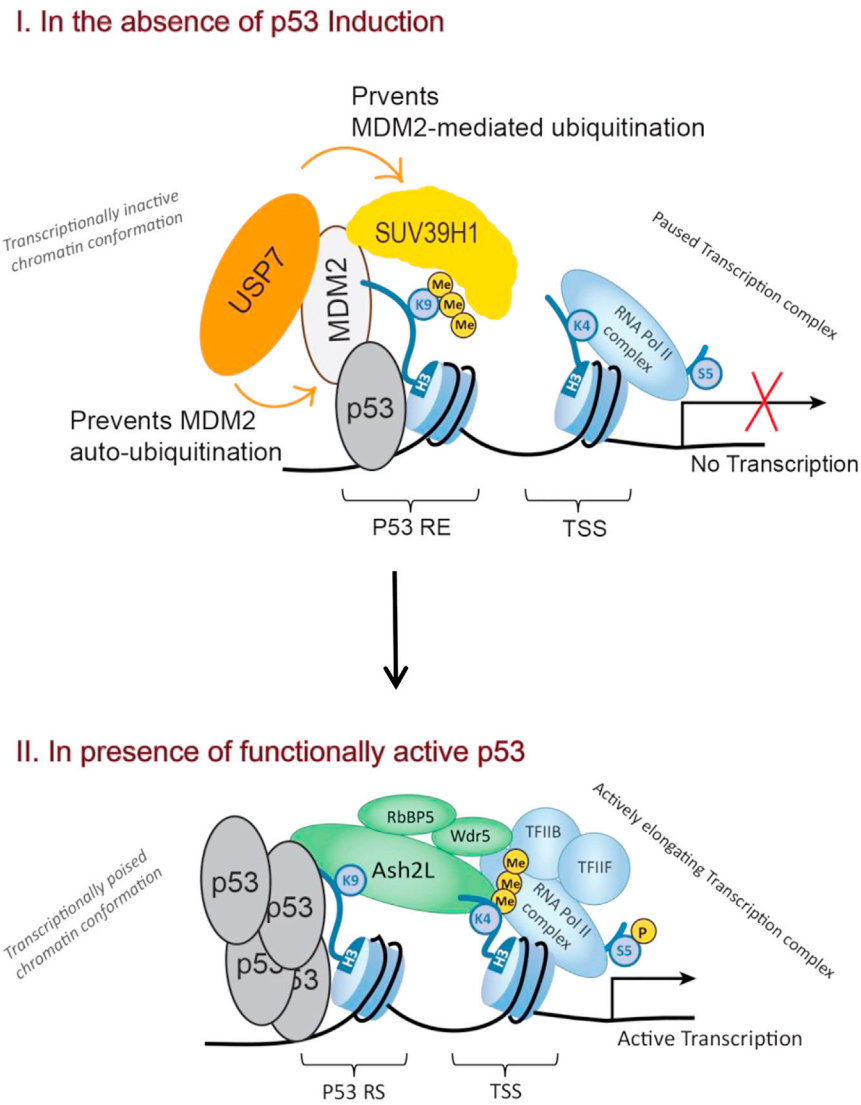

Figure 5. Schematic Diagram Illustrating the Role of USP7 in Maintaining H3K9me3 Mark on p53 Target Promoters

(I) In the absence of p53 induction, "basal" levels of mostly transcriptionally inactive p53 enable the recruitment of the MDM2-USP7-SUV39H1 complex to p53 REs via the p53-MDM2 interaction, placing the repressive mark H3K9me3 and keeping basal transcription activity of the target genes low. USP7 prevents both MDM2 auto-ubiquitination and MDM2-mediated SUV39H1 degradation.

(II) With p53 induction, USP7 promoter occupancy reduces, MDM2 accumulates in the cell, both leading to degradation of SUV39H1 and loss of H3K9me3 mark on p53 target promoters favoring enhanced p53 promoter occupancy, subsequently leading to H3K4me3 enrichment at the transcription start site (Mungamuri et al., 2012, 2014).

Immunoprecipitation Assay

For immunoprecipitation, cells were lysed in immunoprecipitation lysis buffer and precleared with Protein A beads before immunoprecipitation. Protein lysates were incubated with antibody overnight, and the antibody complex was precipitated using Protein A beads. To analyze the role of DNA in protein interactions, the immunoprecipitate was treated with ethidium bromide.

ChIP Assay

ChIP experiments were performed as described previously (Mungamuri et al., 2014). The target sequences were detected by quantitative real-time PCR analysis of eluted DNA. The relative promoter occupancy over the input percentage is shown as a bar diagram. Acetylcholine receptor (AChR) was used as a negative control. Please see Table S1 for the sequences of primers used.

Generation of Stable Cell Lines

HCT116 p53 WT, HCT116 p53$^{-/-}$, and H1299 cells were infected with either Tet-pLKO-shUSP7 or pTripZ: shMDM2 lentivirus and selected for puromycin (2 μg/ml) resistance. Resistant clones were pooled to generate doxycycline-inducible shRNA cell lines.

For further details, please refer to Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, nine figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.049.

AUTHOR CONTRIBUTIONS

S.K.M. and S.A.A. planned the project, and S.K.M. conducted all the experiments. R.F.Q. and S.Y. provided technical support. W.G. and J.J.M. provided advice. S.K.M. and S.A.A. wrote the manuscript.

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