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MDM2 recruitment of lysine methyltransferases regulates p53 transcriptional output

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MDM2 is a key regulator of the p53 tumor suppressor acting primarily as an E3 ubiquitin ligase to promote its degradation. MDM2 also inhibits p53 transcriptional activity by recruiting histone deacetylase and corepressors to p53. Here, we show that immunopurified MDM2 complexes have significant histone H3-K9 methyltransferase activity. The histone methyltransferases SUV39H1 and EHMT1 bind specifically to MDM2 but not to its homolog MDMX. MDM2 mediates formation of p53–SUV39H1/EHMT1 complex capable of methylating H3-K9 *in vitro* and on p53 target promoters *in vivo*. Furthermore, MDM2 promotes EHMT1-mediated p53 methylation at K373. Knockdown of SUV39H1 and EHMT1 increases p53 activity during stress response without affecting p53 levels, whereas their overexpression inhibits p53 in an MDM2-dependent manner. The p53 activator ARF inhibits SUV39H1 and EHMT1 binding to MDM2 and reduces MDM2-associated methyltransferase activity. These results suggest that MDM2-dependent recruitment of methyltransferases is a novel mechanism of p53 regulation through methylation of both p53 itself and histone H3 at target promoters.

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Introduction

The p53 tumor suppressor pathway is functionally altered in the majority of human cancers. It is critical for the maintenance of genomic stability and protection against malignant transformation. P53 is stabilized and activated in response to a variety of stress signals, a property that is essential for its function in development and as a tumor suppressor (Harris and Levine, 2005; Vousden and Lane, 2007). P53 turnover is regulated by MDM2, which binds p53 and functions as an E3 ubiquitin ligase to promote proteasomal-dependent degradation of p53 (Zhang and Xiong, 2001). Mammalian cells also express an MDM2 homolog called MDMX (Shvarts *et al*, 1996). MDMX shares with MDM2 a common primary structure and, just like MDM2, interacts with p53 and inhibits its transcriptional activity.

Knockout of either MDM2 or MDMX in mice results in embryonic lethality because of ectopic activation of p53 (Montes de Oca Luna *et al*, 1995; Parant *et al*, 2001). These experiments have highlighted a critical and non-redundant function for MDM2 and MDMX in the regulation of p53. Moreover, tissue-specific somatic knockout experiments suggest that these proteins have distinct biological functions. Loss of MDMX in different tissues consistently leads to milder phenotypes compared with tissues inactivated for MDM2 (Grier *et al*, 2006; Maetens *et al*, 2007). Biochemically, unlike MDM2, MDMX does not have significant intrinsic E3 ligase activity (Stad *et al*, 2001). Moreover, several ribosomal proteins and ARF specifically bind MDM2 but not MDMX (Wang *et al*, 2001; Gilkes *et al*, 2006), whereas casein kinase 1 α binds specifically MDMX and not MDM2 (Chen *et al*, 2005). Recent studies suggest that the major mechanism of p53 regulation by MDMX is the formation of transcriptionally inactive p53–MDMX complexes, whereas MDM2 primary functions to regulate p53 turnover (Francoz *et al*, 2006; Toledo *et al*, 2006).

MDM2 contains an N-terminal p53-binding domain, a central acidic domain with regulatory functions, and a C-terminal RING domain that confers E3 ubiquitin ligase activity. MDM2 expression is highly inducible by p53, thus forming a negative feedback loop that limits p53 protein levels. DNA damage and mitogenic signals use several mechanisms to induce p53 activation. DNA damage induces phosphorylation of p53 and MDM2 on multiple residues that weaken p53–MDM2 binding and suppresses MDM2 E3 ligase function (Prives and Hall, 1999; Maya *et al*, 2001). Mitogenic signals induce expression of ARF, which binds to MDM2 and prevents MDM2-dependent p53 degradation (Zhang and Xiong, 2001). Ribosomal stress activates p53 by releasing several ribosomal proteins from the nucleolus that bind and inactivate MDM2 (Lohrum *et al*, 2003; Bhat *et al*, 2004; Dai and Lu, 2004; Jin *et al*, 2004).

Although ubiquitination and degradation of p53 is the best-characterized function of MDM2, the ability of MDM2 to interact with other proteins suggests a more sophisticated mode of action. MDM2 binding to the p53 transactivation domain is sufficient to inactivate p53 by displacing coactivators and histone acetyltransferases such as p300 (Teufel *et al*, 2007). Furthermore, MDM2 forms a complex with histone

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deacetylase HDAC1 and inhibits p53 acetylation (Kobet *et al*, 2000; Ito *et al*, 2002; Jin *et al*, 2002). MDM2 also interacts with the nuclear corepressor KAP1, which promotes HDAC1 binding and regulates p53 acetylation (Wang *et al*, 2005). MDM2 contains a putative transcriptional repression domain and is associated with chromatin in a complex with p53 (Thut *et al*, 1997; Jin *et al*, 2002; Arva *et al*, 2005). These observations suggest that MDM2 actively suppress p53 transcriptional activity through the recruitment of various regulatory factors to p53 target promoters.

Chromatin binding by transcription regulators and RNA polymerases is regulated by multiple modifications on core histones (Zhang and Reinberg, 2001). Although acetylation of lysine residues on histone tails are often associated with actively transcribed genes, histone methylation has both positive and negative effects on promoter activity. Histone H3-K4, -K36, -K79 methylation are associated with active transcription, whereas H3-K9 tri-methylation is a hallmark of silenced chromatin. H3-K9 tri-methylation is characteristic of condensed chromatin and transcriptional repression, and is also important for the subsequent establishment of heterochromatin domains by recruitment of HP1 and the associated DNA-methylating enzymes DNMTs (Martin and Zhang, 2005). Abnormal chromatin methylation on histones and DNA are suggested to have critical functions in cancer development by epigenetic silencing of tumor suppressor genes (Baylin and Ohm, 2006).

Several enzymes are involved in the methylation of H3-K9, including SUV39H1, EHMT1 and G9a. SUV39H1 is the human ortholog of the *Drosophila* Su(var)3–9 histone methyltransferase that specifically mediates the tri-methylation of H3-K9 (Aagaard *et al*, 1999; Rea *et al*, 2000). Mice and men also express a second isoform SUV39H2 (O'Carroll *et al*, 2000). Mouse knockout experiments showed that *Suv39h1/2* genes are required for viability and proper H3-K9 tri-methylation in pericentric heterochromatin regions (Peters *et al*, 2001). SUV39H1-null mice are viable but tumor prone (Peters *et al*, 2001). SUV39H1 forms a complex with pRb to inhibit E2F1 transcriptional activity through methylation of E2F1 target promoters (Nielsen *et al*, 2001). Rb-mediated recruitment of SUV39H1 to the E2F targets may be required for the formation of senescence-associated heterochromatin foci (Narita *et al*, 2003). However, H3-K9 methylation and HP1 recruitment also occurs in transcribed regions and may have a function in promoting transcription elongation by suppressing cryptic initiation (Vakoc *et al*, 2005; Berger, 2007). Therefore, compelling evidence indicate that SUV39H1 is involved in the regulation of H3-K9 methylation at heterochromatin but also, to a lesser extend, at euchromatin domains.

EHMT1 (also called GLP) is a close relative to G9a (Ogawa *et al*, 2002). EHMT1 and G9a function as heterodimers to mediate H3-K9 methylation at euchromatin (Tachibana *et al*, 2005). Knockout mice have been generated for G9a and EHMT1. The phenotypes for EHMT1 and G9a deficiency are nearly identical, including very early embryonic lethality, drastic reduction of H3-K9 mono- and di-methylation, and HP1 relocalization (Tachibana *et al*, 2002, 2005). EHMT1 and G9a are thus critical for H3-K9 mono- and di-methylation at euchromatin. This function is distinct from the function of SUV39H1, which is mainly involved in H3-K9 tri-methylation at heterochromatin sites.

Recent studies showed that p53 is positively regulated by mono-methylation of K372 by the SET9 methyltransferase (Chuikov *et al*, 2004), negatively regulated by mono-methylation of K370 by Smyd2 (Huang *et al*, 2006), positively regulated by di-methylation of K370 by an unknown enzyme (Huang *et al*, 2007), and negatively inhibited by mono-methylation of K382 by SET8 (Shi *et al*, 2007). Furthermore, di-methylation of R333, R335, and R337 by PRMT5 alters the promoter selectivity of p53 and favours cell cycle arrest over apoptosis (Jansson *et al*, 2008). In this report, we show that MDM2 specifically interacts with histone methyltransferase SUV39H1 and EHMT1. MDM2 mediates the formation of p53–MDM2–SUV39H1/EHMT1 complex capable of methylating histone H3-K9, which may account for a paradoxical rise of H3-K9 methylation level during p53 activation. Furthermore, MDM2-dependent recruitment of EHMT1 promotes mono-methylation of p53 at K373. Our results suggest that MDM2–SUV39H1/EHMT1 interactions have a significant function in p53 regulation by methylating histone H3 at p53 target promoters, and to a lesser extent methylating p53 itself.

Results

MDM2-containing complexes possess histone methyltransferase activity

MDM2 may directly inhibit p53 transcriptional activity through its ability to interact not only with transcriptional repressors such as YY1 (Sui *et al*, 2004) and Kap1 but also with histone methyltransferases. To test whether MDM2-containing complexes possess histone methyltransferase activity, MDM2 was immunoprecipitated from stably transfected H1299 cells and incubated with ^{3}H-SAM and GST fusion containing N-terminal 56 amino acids of histone H3 (GST-H3). Significant methylation of GST-H3 was detected in the MDM2-immunopurified complexes on MG132 exposure (Figure 1A). This treatment was used to block proteasome-dependent degradation of MDM2. Control H1299 cells express very low levels of endogenous MDM2 and showed low background activity. Immunocomplexes of endogenous MDM2 from SJSA cells, a cell line with MDM2 gene amplification, also showed significant activity on MG132 exposure or induction of MDM2 expression using the p53 activator Nutlin (Vassilev *et al*, 2004) (Figure 1A). Bacteria expressed GST-MDM2 had no activity (data not shown), indicating that the methylation activity was likely because of the presence of cellular methyltransferases in the coprecipitates.

MDMX is also an important regulator of p53 transcriptional activity. In transient transfection assays, MDMX IP showed much lower methyltransferase activity compared with MDM2 despite high level of expression (Figure 1B). P53 IP showed weak but detectable activity, which is likely because of interaction with endogenous MDM2 (see below). Furthermore, in contrast to MDM2, MDMX does not seem to interact with methylation enzymes in direct coimmunoprecipitation assays (see Figure 3B). These results indicate that MDM2 but not MDMX is specifically found in complexes together with methyltransferases.

MDM2-containing complexes specifically methylate histone H3 lysine 9

To further test the substrate specificity of MDM2-associated methyltransferase activity, MDM2 immunoprecipitated from different cell lines were used in an *in vitro* methylation assay

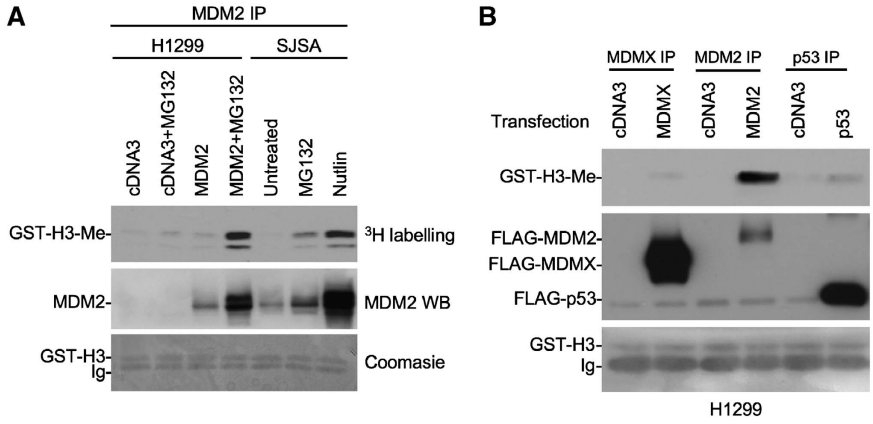

Figure 1 MDM2 complex has histone methyltransferase activity. (**A**) MDM2 was immunoprecipitated from H1299 cells stably transfected with MDM2 or from SJSA cells with amplified MDM2 using antibody 5B10. Cells were treated with 30 μM MG132 for 4 h or with 8 μM Nutlin for 16 h to increase MDM2 level. The MDM2 complex was incubated with ^{3}H-SAM and GST-histone H3-1-56 fusion protein. Methylation of GST-H3 was detected by autoradiography. (**B**) H1299 cells transiently transfected with FLAG-tagged MDM2, MDMX, and p53 were immunoprecipitated using indicated antibodies and analysed for methylation of GST-H3. Relative protein expression levels were determined by anti-FLAG western blot.

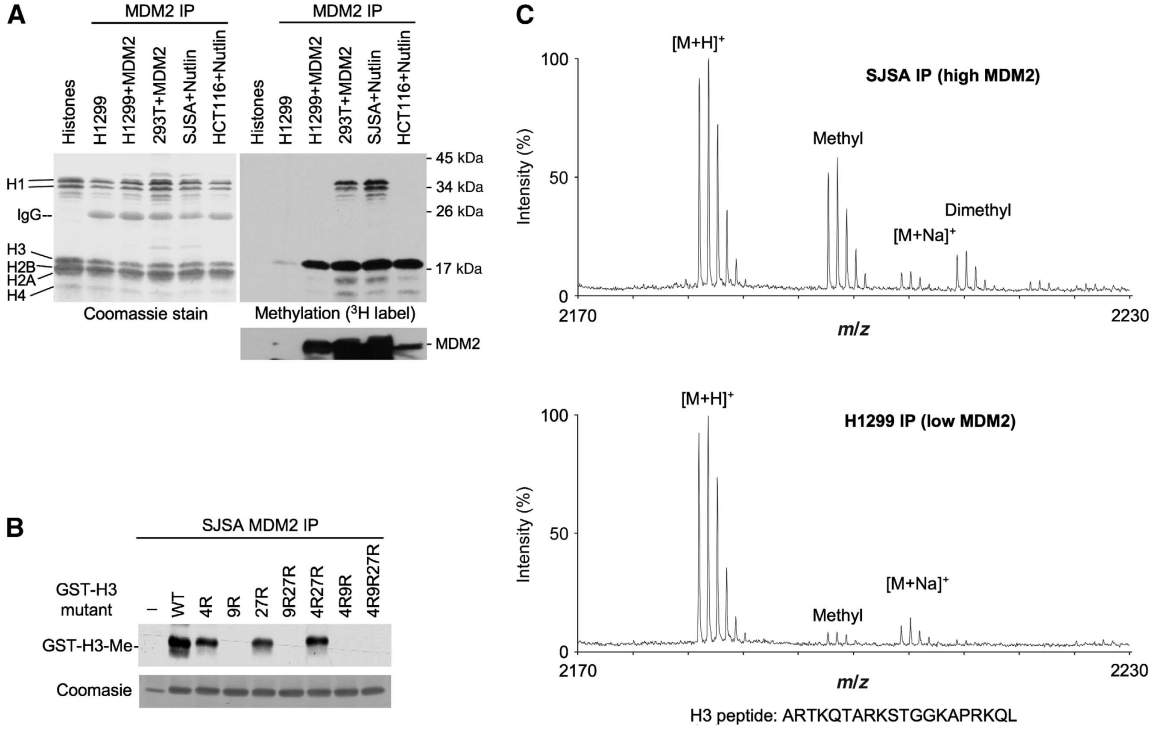

Figure 2 MDM2 associates with H3-K9-specific methyltransferase. (**A**) Endogenous and transfected MDM2 was immunoprecipitated from indicated cell lines and incubated with ^{3}H-SAM and a mixture of core histones. The reaction products were fractionated by SDS–PAGE and transferred to membrane. The membrane was exposed against film (right panel), stained with Coomassie blue (left panel), and probed for MDM2 level (bottom panel). (**B**) MDM2 immunoprecipitated from SJSA cells were incubated with GST-H3-1-56 with K-to-R mutations at indicated sites and ^{3}H-SAM. The results suggest that K9 is the target of MDM2-associated methylases. (**C**) H1299 (low MDM2 level) and SJSA (high MDM2 level) were immunoprecipitated using MDM2 antibody and incubated with histone H3 peptide (aa 1–20) and SAM. The reaction products were analysed by mass spectrometry and the peptides mono-methylated ($\Delta m/z = 14$) and di-methylated by MDM2 ($\Delta m/z = 28$) were indicated. Fragmentation analysis of the mono-methylated peptide indicated that K9 was modified (not shown).

using a mixture of histones (H1, H2A, H2B, H3, H4) as substrates. The results showed that MDM2-containing complexes predominantly methylate histone H3 (Figure 2A), although weak methylation of H1 was also detected at high MDM2 levels. To identify the histone H3 residue modified by the MDM2 complex, GST-H3 mutants on various lysine residues were used as substrate in the *in vitro* methylation reaction. The results indicate that K9 is the target of MDM2-dependent methylation (Figure 2B). To further confirm the methylation site, histone H3 and H4 peptides were used as substrates for the *in vitro* reaction using non-radioactive SAM. The reaction products were analysed by mass spectrometry. The MDM2-containing complexes specifically methylated the H3 peptide but not the H4 peptide. The *in vitro*

reaction generated mostly mono- and di-methylated H3 peptides (Figure 2C). Fragmentation analysis of the methylated H3 peptide produced Y ions that were indicative of K9 methylation (data not shown). We conclude that MDM2 coprecipitates with methyltransferase specific for histone H3-K9.

MDM2 forms a complex with SUV39H1 and EHMT1

To identify the histone methyltransferase responsible for the MDM2-associated enzymatic activity, the ability of MDM2 to bind to several enzymes known to target H3-K9 was tested by coimmunoprecipitation. FLAG-tagged histone methyltransferases and controls (Chk2 and KAP1) were cotransfected with MDM2 and immunoprecipitated using a FLAG antibody. The immunoprecipitates were resolved in denaturing gels and analysed by western blot using MDM2-specific antibody. Use of FLAG-tagged enzymes enabled us to compare the relative binding efficiency of each enzyme to MDM2. The results showed that SUV39H1 and EHMT1 bind significantly to MDM2 (Figure 3A). In contrast, MDMX interaction with the methyltransferases is negligible (Figure 3B), whereas its interaction with Chk2 (which phosphorylates MDMX) and KAP1 used here as positive controls could be detected in the same experimental conditions. These results are consistent with the lack of methyltransferase activity in MDMX IP (Figure 1B). The p53-modifying enzyme SET9 did not interact with MDM2 (Figure 3A). These results indicate that MDM2, but not MDMX, interacts with histone methyltransferase. Among the enzymes analysed, SUV39H1 and EHMT1 showed the highest affinity for MDM2. In separate experiments, the EHMT1 heterodimeric partner G9a also showed significant binding to MDM2 (Figure 3C).

When cell lysates were immunoprecipitated using SUV39H1 or EHMT1 antibodies and probed using MDM2 antibody, coprecipitation of MDM2 with endogenous SUV39H1 and EHMT1 were detectable in cells with high-level MDM2 (SJSA) (Figure 3D). This result indicates that the interactions between MDM2 and SUV39H1 or EHMT1 occur at endogenous levels of expression. Furthermore, using a separate approach, we found that MDM2 immunoprecipitates

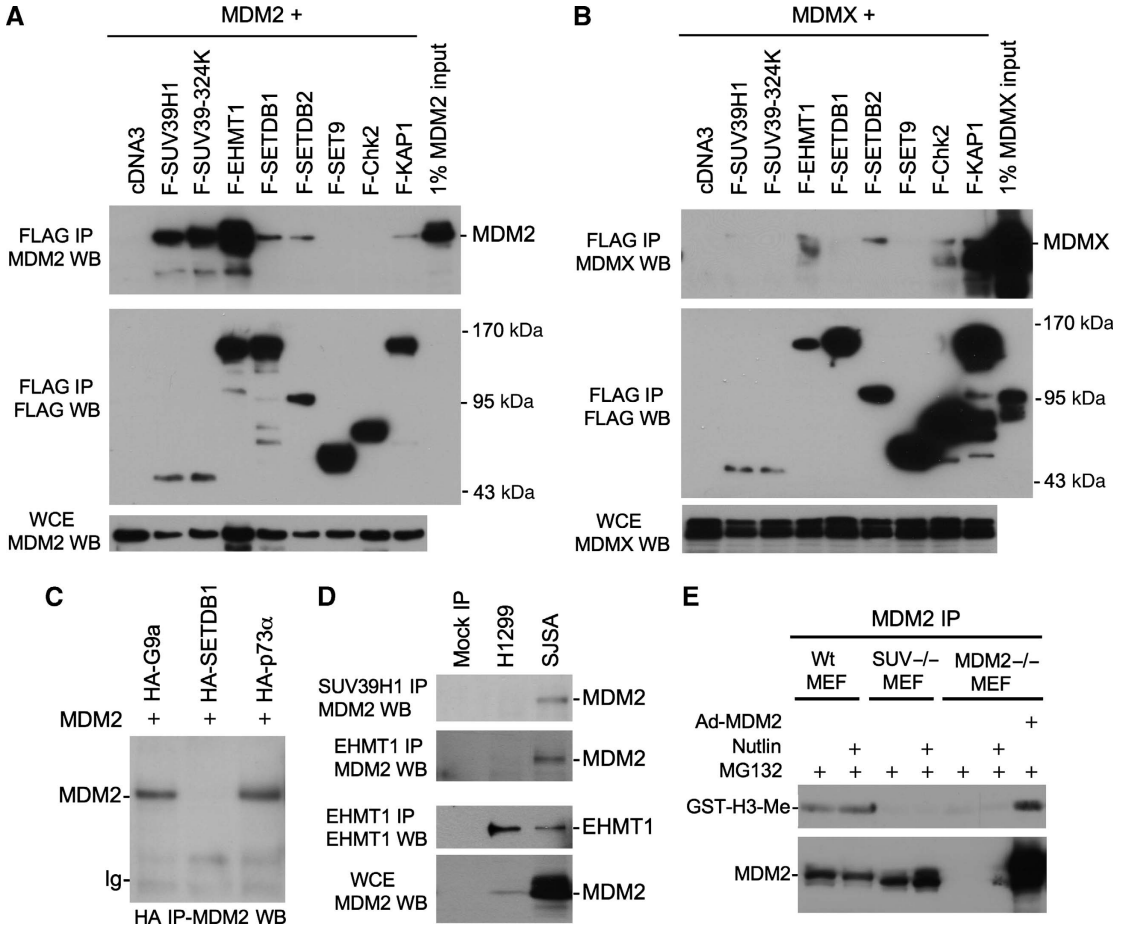

Figure 3 MDM2 specifically interacts with EHMT1 and SUV39H1. (**A**) MDM2 and (**B**) MDMX were transfected with FLAG-tagged enzymes into H1299 cells. Cells were immunoprecipitated using FLAG antibody and the coprecipitated MDM2 and MDMX were detected by western blot. The filters were probed with rabbit anti-FLAG antibody to determine the levels of enzyme expression. MDM2 and MDMX expression levels were confirmed by western blot of whole-cell extract (WCE). SUV39H1-324 K is a catalytically inactive mutant. (**C**) H1299 cells transiently transfected with MDM2 and indicated HA-tagged proteins were analysed by HA IP followed by MDM2 western blot to detect the coprecipitation of MDM2. HA-p73 serves as positive control for MDM2 binding. (**D**) Binding of endogenous MDM2, SUV39H1 and EHMT1 in SJSA cells were detected by IP using SUV39H1 and EHMT1 antibodies followed by MDM2 western blot. (**E**) MDM2 was immunoprecipitated from MEFs derived from SUV39H1/H2 double-null mice and analysed for methyltransferase activity using GST-H3 as substrate. MDM2-null MEF infected with MDM2 adenovirus served as control.

from Suv39h1/2 double-null MEFs contained significantly lower methyltransferase activity compared with MDM2 complexes from wild-type MEFs (Figure 3E). Endogenous Suv39h1 or Suv39h2 are therefore significant mediators of the MDM2-associated methyltransferase activity in MEFs. As EHMT1 levels are variable in different cell lines (e.g. very low in MEFs, Supplementary Figure S3B), the relative contributions of SUV39H1 and EHMT1 to MDM2-associated methylase activity are likely to be cell type dependent.

Given the ability of MDM2 to interact with SUV39H1, it was somewhat surprising that MDM2 IP did not produce tri-methylated H3 peptide *in vitro* (Figure 2C). Our control experiments using purified FLAG-SUV39H1 showed that high levels of SUV39H1 were needed to produce tri-methylated H3 peptide in the *in vitro* reaction (data not shown), which was not attainable by MDM2 co-IP of endogenous SUV39H1. In the presence of low levels of SUV39H1, the primary products were mono- and di-methylated H3 peptide because of the sequential nature of the reaction.

Mapping of MDM2 and SUV39H1 interaction domains

To determine the domain of SUV39H1 that interacts with MDM2, FLAG-tagged deletion mutants of SUV39H1 were constructed and cotransfected with MDM2 in H1299 cells. IP-western blot assay show that a region of SUV39H1 that includes the nuclear localization signal and pre-SET domain is critical for MDM2 binding (Supplementary Figure S1). This binding evidently has a recruiting function and does not affect SUV39H1 enzymatic activity, as shown below.

Using a panel of MDM2 deletion mutants, we found that SUV39H1 and EHMT1 interact with the MDM2 acidic domain (Supplementary Figure S2). On the basis of the fact that MDMX does not bind SUV39H1 and EHMT1 (Figure 2B), a panel of MDM2–MDMX hybrid constructs were also tested, which resulted in the same conclusions (data not shown) (Kawai *et al*, 2003). These results further expand the repertoire of MDM2 acidic domain as a multi-functional protein-binding region.

MDM2 mediates the formation of active p53–SUV39H1/EHMT1 complexes

We next tested whether MDM2 promotes the binding of SUV39H1 and EHMT1 to p53, which is needed for their recruitment to p53 target promoters. To this end, p53 was cotransfected with MDM2, SUV39H1, and EHMT1. P53 was immunoprecipitated with Pab1801 and tested for its ability to methylate GST-H3. A longer exposure showed that p53 IP contain weak but readily detectable methyltransferase activity for GST-H3 (Figure 4A). SUV39H1 and EHMT1 were not efficiently coimmunoprecipitated with p53, but cotransfection of MDM2 resulted in efficient p53–SUV39H1 and p53–EHMT1 coprecipitation. This observation indicates that MDM2 may act as abridging molecule to allow the formation of trimeric complexes. The p53-associated methyltransferase activity was also significantly stimulated after coexpression of SUV39H1/EHMT1 and MDM2 (Figure 4A). These results indicate that MDM2 mediates complex formation between p53, SUV39H1, and EHMT1. Furthermore, the p53-containing complexes have the ability to methylate histone H3. Quantitatively, SUV39H1 and EHMT1 enzymatic activity were fully retained after interaction with MDM2 and p53 (Figure 4A, compare p53 IP and MDM2 IP). The critical function of MDM2 acidic domain in mediating trimeric complex formation was further confirmed using the 1–200

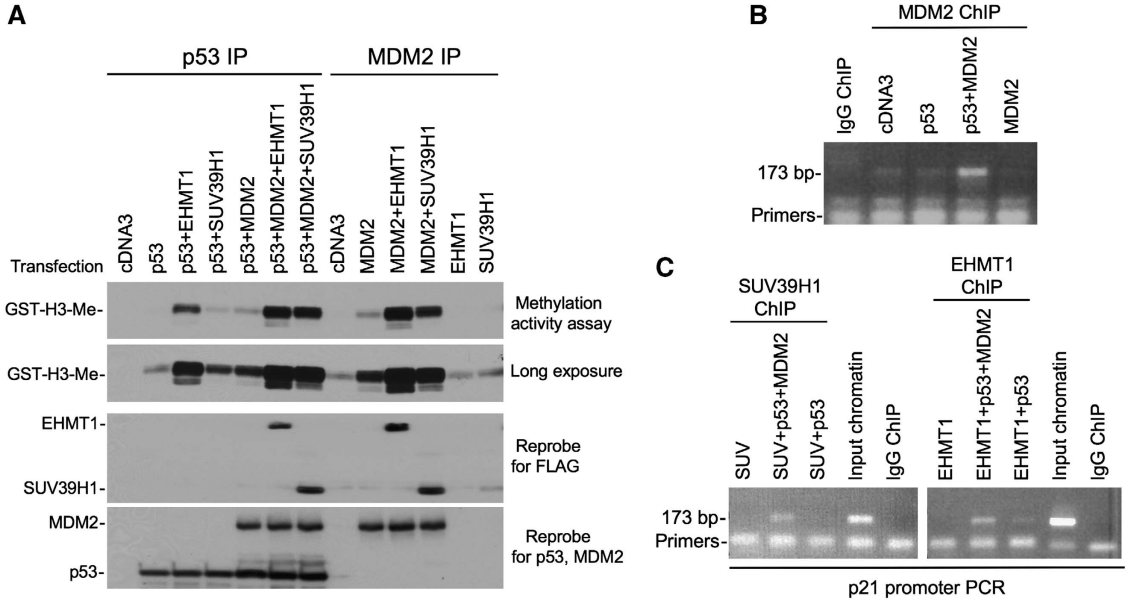

Figure 4 MDM2 mediates formation of active p53–SUV39H1/EHMT1 complex. (**A**) H1299 cells were transfected with p53, MDM2, FLAG-SUV39H1, and FLAG-EHMT1. P53 and MDM2 IPs were analysed for GST-H3 methylation activity. Coprecipitation of SUV39H1 and EHMT1 was confirmed by probing the p53 and MDM2 IP using FLAG antibody. P53 alone did not coprecipitate SUV39H1 or EHMT1. Coexpression of MDM2 stimulated p53-SUV39H1/EHMT1 binding and increased p53-associated methyltransferase activity. (**B**) MDM2 is recruited to DNA by p53. H1299 cells were transfected with p53 and MDM2 for 24 h and analysed by ChIP using anti-MDM2 antibody and PCR detection of the p21 promoter DNA. (**C**) Promoter recruitment of SUV39H1 and EHMT1 by MDM2 and p53. H1299 cells were transfected with p53, MDM2, Myc-SUV39H1, and Myc-EHMT1 for 24 h. Cells were analysed by chromatin immunoprecipitation using Myc antibody and PCR detection of the p21 promoter DNA.

fragment of MDM2 that does not have the acidic domain (Supplementary Figure S3A).

As reported earlier (Jin *et al*, 2002; Minsky and Oren, 2004; White *et al*, 2006), MDM2 can be recruited to promoters by p53 as shown by chromatin immunoprecipitation (ChIP) assay (Figure 4B). To test whether MDM2 in turn recruits methyltransferases to p53 target promoters, H1299 cells were transfected with p53, MDM2, SUV39H1, and EHMT1. SUV39H1 and EHMT1 recruitment to the p21 promoter was analysed by ChIP. The results showed that in the absence of p53, SUV39H1, and EHMT1 did not bind to the p21 promoter. P53 expression led to weak binding of EHMT1 to p21 promoter. Importantly, MDM2 expression stimulated SUV39H1 and EHMT1 binding to the p21 promoter (Figure 4C). These results showed that MDM2 recruits SUV39H1 and EHMT1 to p53-responsive promoter and may repress p53-mediated transcription by methylating histone H3.

MDM2 stimulates EHMT1 methylation of p53 K373

To test whether MDM2-dependent recruitment of SUV39H1 and EHMT1 promotes methylation of p53 itself, p53 was cotransfected with MDM2, SUV39H1, and EHMT1 and immunoprecipitated using specific p53 antibody. The p53 complexes were incubated with ^{3}H-SAM. The data showed that p53 was methylated by EHMT1 *in vitro* (Figure 5A). Furthermore, MDM2 stimulated EHMT1–p53 interaction and increased p53 methylation (Figure 5A). The E3 ligase deficient MDM2-457S mutant retained its ability to promote p53 methylation. The EHMT1 dimeric partner G9a also showed significant p53-methylation activity, which was further stimulated by high levels of MDM2-457S (Figure 5A). Despite efficient recruitment of SUV39H1 by MDM2, p53 was not methylated by SUV39H1 (Figure 5A). We also did not observe MDM2 methylation by EHMT1 or SUV39H1 in these experiments despite high MDM2 levels in the IP complex (data not shown). We conclude that MDM2 specifically promotes EHMT1- and G9a-mediated p53 methylation.

The ability of MDM2 to stimulate p53 methylation by EHMT1 was also tested using a titration assay. Purified EHMT1-methylated His6-p53 *in vitro*, suggesting that the reaction can occur in the absence of MDM2 (Figure 5B). However, purified EHMT1–MDM2 complex showed 4-fold higher activity in methylating p53 compared with EHMT1 alone. EHMT1 self-methylation was not affected by MDM2 binding, suggesting that MDM2 does not alter EHMT1 catalytic activity (Figure 5B). The data therefore favour a mechanism in which stimulation of p53 methylation by MDM2 depends on its ability to recruit EHMT1 to p53 rather than to stimulate its catalytic activity.

EHMT1 did not methylate GST-p53 with C-terminal deletions (Figure 6A) or K-to-R mutation of nine C-terminal lysine residues (Figure 6B). These results indicate that EHMT1 methylates p53 on C-terminal lysines. To more precisely identify the methylation site, single K-to-R mutants of p53 were tested for methylation by the EHMT1–MDM2 complex. The results showed that K373 is critical for the modification (Figure 6B). Similar analysis indicated that G9a methylation of p53 also required K373 (data not shown). Therefore, MDM2 recruitment of EHMT1 stimulates p53 methylation specifically at K373.

To show that p53 K373 methylation occurs *in vivo*, antibodies were raised against p53 peptides with mono-methylated or di-methylated K373 (K373Me1, K373Me2). Although no specific signal was obtained using the K373Me2 antibody, the K373Me1 antibody showed increased reactivity to p53 after coexpression of EHMT1. Furthermore, MDM2 stimulated p53 K373 methylation in the presence of exogenous EHMT1 (Figure 6C). The antibody showed reasonable specificity with little cross-reactivity to recombinant p53 and p53-373R mutant. Consistent with the ability of MDM2 to stimulate p53 methylation in transfection assays, endogenous p53 coprecipitated with MDM2 showed higher levels of K373 methylation than total p53 (Figure 6D). These results indicate that MDM2 regulates p53 K373 methylation *in vivo*. However, the relatively weak signal suggests that the fraction of p53 being methylated on K373 is small.

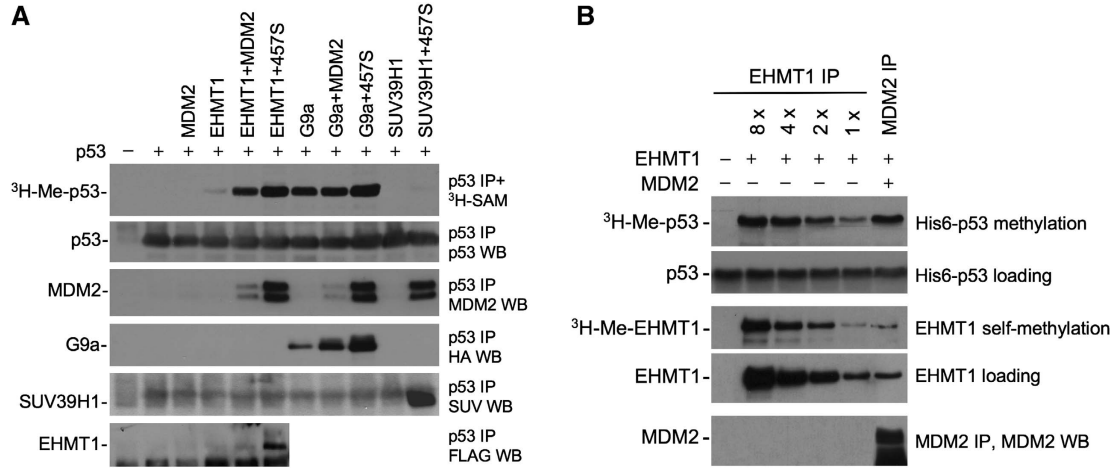

Figure 5 MDM2 stimulates p53 methylation by EHMT1. (**A**) H1299 cells were transfected with indicated plasmids. P53 was immunoprecipitated and incubated with ^{3}H-SAM. P53 methylation was detected by autoradiography, and coprecipitated proteins were detected by western blot. (**B**) FLAG-EHMT1 was transfected alone or with MDM2 into H1299 cells. EHMT1 was purified by anti-FLAG IP; MDM2–EHMT1 complex was purified by anti-MDM2 IP and analysed for methylation of His6-p53. EHMT1 was used at different amounts to compare its methyltransferase activity to MDM2–EHMT1 complex.

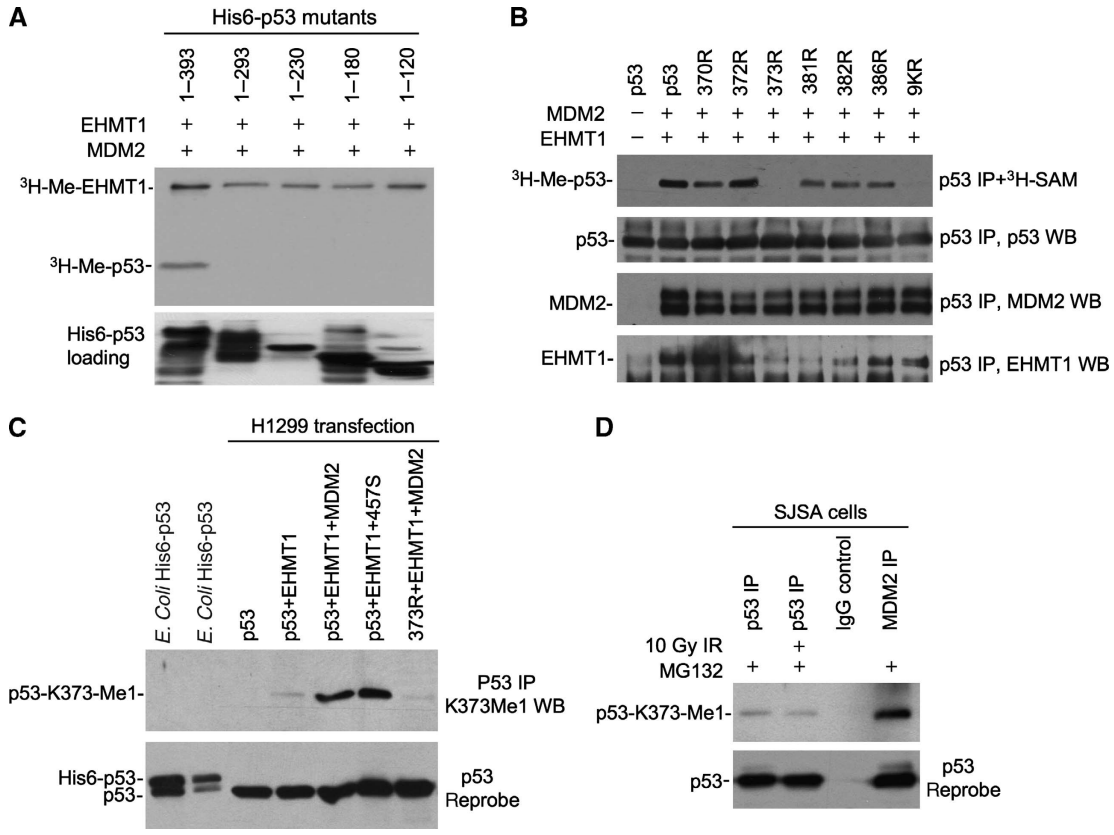

Figure 6 MDM2 stimulates p53 K373 methylation by EHMT1. (**A**) MDM2–EHMT1 complex was precipitated from transfected H1299 cells using MDM2 antibody and incubated with recombinant His6-p53 deletion mutants and ^{3}H-SAM. P53 methylation was detected by autoradiography. (**B**) H1299 cells were transfected with p53 point mutants, MDM2, and EHMT1. P53 was immunoprecipitated and incubated with ^{3}H-SAM. P53 methylation was detected by autoradiography and western blot. (**C**) H1299 cells were transfected for 24 h with p53, MDM2, and EHMT1. P53 was immunoprecipitated with Pab1801 and probed with K373Me1 antibody. The membrane was stripped and reprobed for the level of total p53. (**D**) SJSA cells were immunoprecipitated using MDM2 antibody 5B10 or p53 antibody Pab1801. Samples containing similar levels of MDM2-bound p53 or total p53 were probed with K373Me1 antibody, and reprobed with p53 antibody.

SUV39H1 and EHMT1 inhibit p53 transcriptional activity

The functional consequences of SUV39H1 and EHMT1 binding to p53 were analysed using p53 reporter gene assays. Cotransfection of SUV39H1 and p53 into MDM2/p53 double-null MEFs had no effect on the activation of p53-responsive reporter BP100-luc. However, coexpression of MDM2 and SUV39H1 cooperatively inhibited p53 activity. Furthermore, the inactive SUV39H1-H324K mutant did not inhibit p53 (Figure 7A), indicating that the effect of SUV39H1 on p53 was dependent on its methyltransferase activity.

Similar to SUV39H1, EHMT1 also inhibited p53 activity in an MDM2-dependent manner (Figure 7B). Furthermore, EHMT1 inhibited the p53-373R mutant, although less efficiently than wild-type p53 (Figure 7B). Therefore, both SUV39H1 and EHMT1 cooperate with MDM2 to inhibit p53 transcriptional activity. Methylation of p53 K373 was not absolutely required for repression by EHMT1, but partially contributed to the effect. Together, these results are consistent with a model in which SUV39H1 and EHMT1 are recruited to the DNA-bound MDM2–p53 complexes and subsequently inhibit transcription by methylating chromatin.

To further test this possibility, we attempted to generate U2OS cell lines with constitutive expression of SUV39H1 and EHMT1. Although no cell line with ectopic EHMT1 expression could be obtained (not shown), clonal cell lines expressing high level SUV39H1 could be established (Figure 7C). However, these cells rapidly lose SUV39H1 expression after few passages in culture (not shown). When early passage SUV39H1 cell lines were analysed for p53 activity, p53-dependent induction of MDM2 and p21 in response to actinomycin D exposure was significantly impaired (Figure 7D). Overexpression of the inactive SUV39H1-H324K mutant was tolerated in long-term culture and did not affect p53 response (Figure 7D). These results indicate that SUV39H1 overexpression dampens stress-induced p53 transcriptional activity.

P53 activation is associated with increased promoter H3-K9 methylation

To determine whether H3-K9 methylation levels at p53-responsive promoters change during stress response, SJSA cells were treated with IR and analysed by ChIP assay using antibodies against p53, H3-K9-Me3, H3-K9-Me2, and H3-K9-Ac. The results showed that IR stimulated p53 binding to p21 promoter and significantly increased K9-Ac level, consistent with promoter activation (Figure 8A). Surprisingly, K9-Me2 and K9-Me3 levels at the p21 promoter were also reproducibly increased after p53 activation, although affecting a lower percentage of promoters than acetylation (Figure 8A). Similar results were also observed at the p53 target PUMA promoter (data not shown). This effect was specific for p53-responsive

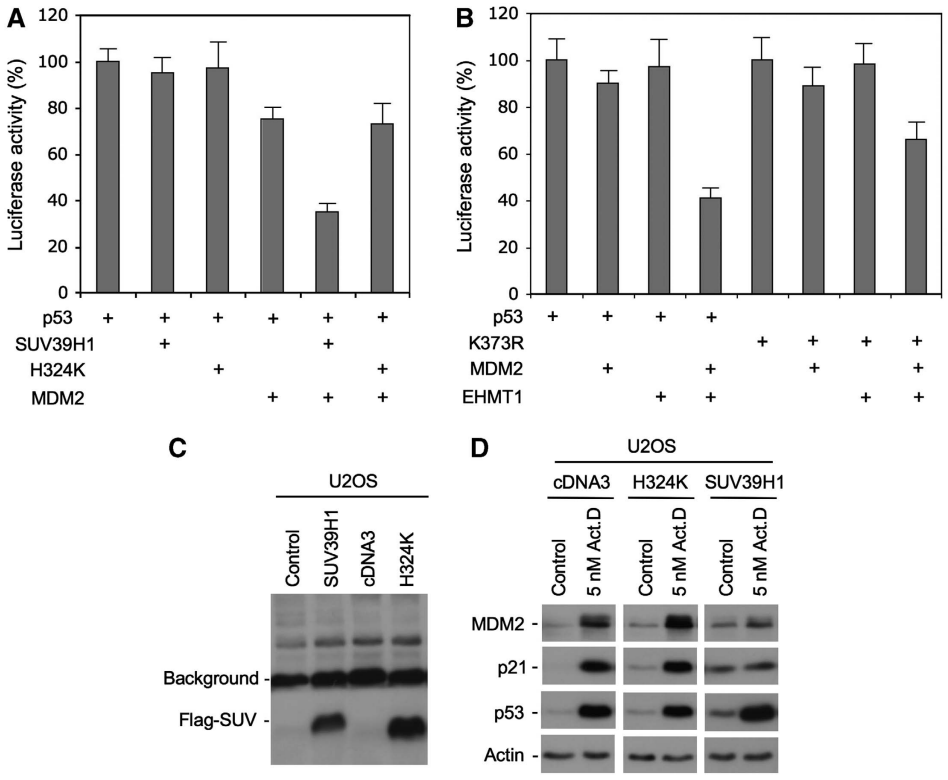

Figure 7 EHMT1 and SUV39H1 inhibit p53 transcriptional activity. (**A**) MDM2/p53-null MEFs were transfected with p53-responsive reporter BP100-luc and indicated plasmids. P53 activity was determined after 24 h by luciferase assay. SUV39H1-H324K is an inactive mutant for control. (**B**) MDM2/p53-null MEFs were transfected with BP100-luc reporter, wt p53 and p53-373R mutant resistant to methylation by EHMT1. P53 activity was determined after 24 h by luciferase assay. (**C**) U2OS cells were transfected with wt and mutant SUV39H1 and clonal cell lines were identified by western blot and immunofluorescence staining to confirm uniform expression (not shown). (**D**) U2OS cell lines expressing wt and mutant SUV39H1 were treated with 5 nM actinomycin D for 18 h and analysed for p53 target gene expression by western blots.

genes as H3-K9 modifications at an unrelated DNA sequence (Sat2 repetitive element) were not affected (Figure 8B).

To further test whether MDM2 promotes increase of K9 methylation at the p21 promoter, U2OS expressing tetracycline-repressible MDM2 was analysed. The result showed that expression of MDM2 increased the basal level of K9-Me3 at the p21 promoter (Figure 8C). Stress treatments that induce p53 and MDM2 also further increased K9-Me3 level at the p21 promoter similar to SJSA cells (Figure 8C and D). Further attempts to detect increased recruitment of SUV39H1 and EHMT1 to p21 promoter after stress were not informative because of insufficient signal. These results are consistent with MDM2–SUV39H1/EHMT1 complex acting as a negative feedback regulator to dampen p53 target activation during stress response. The rise of p53 and MDM2 levels during stress is expected to result in more p53–MDM2–SUV39H1 complexes and a higher frequency of methylating p53 target promoters.

Endogenous SUV39H1 and EHMT1 regulate p53 stress response

The paradoxical rise of repressive H3-K9 modifications at p53 target promoters during p53 activation suggests that eliminating SUV39H1 and EHMT1 may reduce MDM2-mediated negative feedback. Therefore, stress-induced p53 activity was examined in U2OS cells depleted for SUV39H1 by siRNA. SUV39H1 knockdown significantly enhanced p53-dependent induction of p21 and MDM2 expression in response to actinomycin D treatment (Figure 9A). Endogenous SUV39H1 can therefore function as a negative regulator of stress-induced p53.

Similarly, transient knockdown of EHMT1 by siRNA also leads to increased MDM2 and p21 expression in U2OS cells in response to ribosomal stress (Figure 9B) and DNA damage (Figure 9C). To confirm that the effect of EHMT1 siRNA on p53 target gene expression was due to activation of p53 rather than a global derepression, we used U2OS cells expressing the HPV E6 oncoprotein. E6-mediated degradation of p53 abrogated the induction of MDM2 and p21 by DNA damage and EHMT1 siRNA (Figure 9C). These results show that both SUV39H1 and EHMT1 are able to modulate p53 transcriptional activity. These results were also confirmed at the mRNA level by RT–PCR analysis of p53 target genes (Supplementary Figure S4).

Importantly, knockdown of SUV39H1 and EHMT1 sensitized U2OS cells to p53-mediated cell cycle arrest after IR treatment (Figure 9D). Furthermore, the relevance of SUV39H1-dependent regulation of p53 was assessed in a model of p53-induced cellular senescence. Normal human diploid BJ fibroblasts exposed to Nutlin undergo p53-dependent senescence as revealed by a pronounced cell cycle arrest and SA-β-Gal-positive staining (Figure 9D and data not shown). BJ cells with stable knockdown of SUV39H1 and p53 were generated using shRNA expression vectors (Figure 10A and B). Similar to tumor cells, SUV39H1 knockdown in non-transformed fibroblasts increased p53-mediated

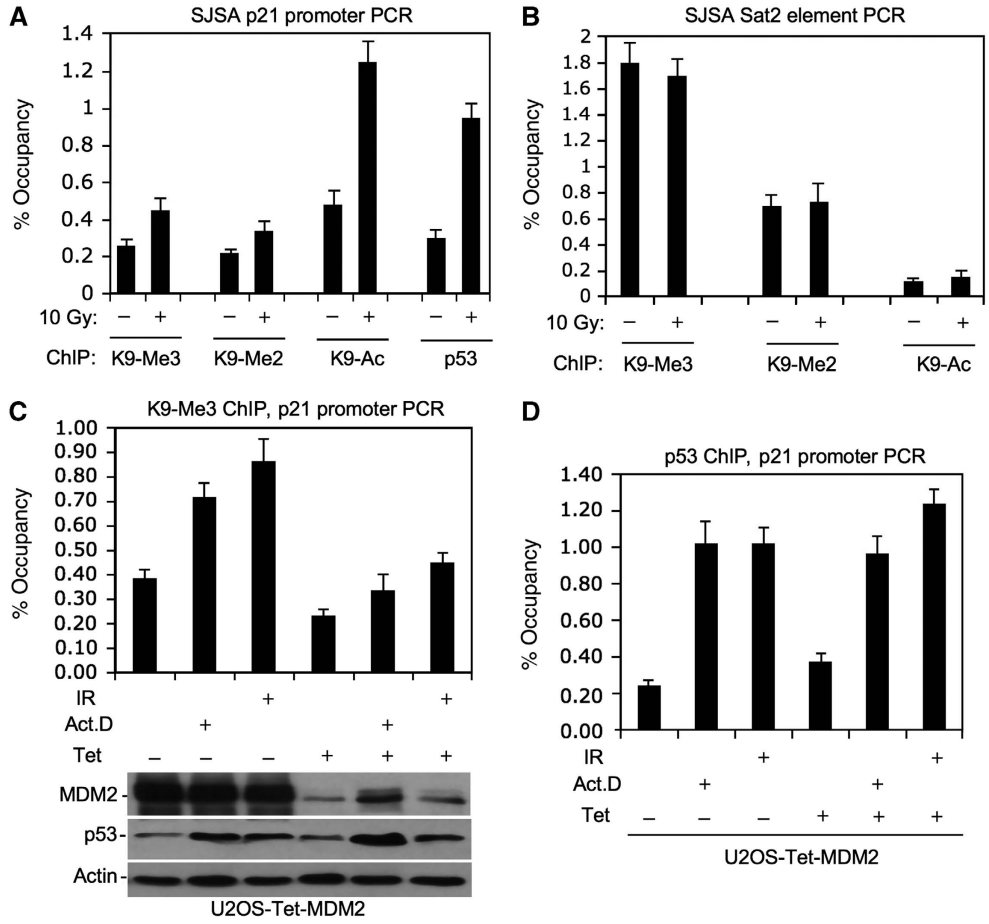

Figure 8 EHMT1 and SUV39H1 inhibit p53 transcriptional activity. (**A**, **B**) SJSA cells were treated with 10 Gy irradiation and processed for ChIP using indicated antibodies after 16 h. The precipitated chromatin were analysed using primers for p21 promoter and the Sat2 repetitive element. (**C**, **D**) U2OS cells stably expressing Tet-off MDM2 were treated with 1 μg/ml tetracycline for 24 h to repress MDM2 expression, and irradiated with 10 Gy IR or treated with 5 nM actinomycin D for 18 h. Cells were analysed by ChIP using H3-K9-Me3 and p53 antibodies and p21 promoter PCR. Duplicate samples were analysed by western blot.

p21 induction in response to Nutlin-3a and DNA damage (Figure 10C and data not shown). Importantly, SUV39H1 knockdown significantly reduced cell proliferation in a Nutlin dose-dependent manner (data not shown) and accelerated the appearance of SA-β-Gal-positive cells (Figure 10D and E). These results indicate that SUV39H1 functions as a negative regulator of p53-induced cellular senescence.

It is noteworthy that overexpression or knockdown of SUV39H1 and EHMT1 did not affect p53 protein levels before and after stress (Figures 7D and 9A–C). Furthermore, SUV39H1 and EHMT1 expression did not affect the ability of MDM2 to promote p53 ubiquitination and degradation in transfection assays (data not shown). Therefore, MDM2 interactions with SUV39H1 and EHMT1 lead to regulation of p53 transcriptional activity but not p53 turnover.

ARF inhibits MDM2 interactions with SUV39H1 and EHMT1

Deletion mapping experiments showed that SUV39H1 and EHMT1 interact with the MDM2 acidic domain (Supplementary Figure S2). ARF is an important activator of p53 by binding to MDM2 acidic domain and inducing p53 stabilization. Therefore, we tested whether ARF disrupts MDM2 interaction with methyltransferases, which may help activate p53 function. Cotransfection experiments show that ARF expression significantly inhibited MDM2 binding to SUV39H1 and EHMT1 (Figure 11A). Furthermore, ARF inhibited the ability of MDM2 to recruit EHMT1 to p53, and inhibited MDM2-dependent p53 methylation *in vitro* (Figure 11B).

To test whether endogenous MDM2-associated methyltransferase activity is regulated by ARF, U2OS cells expressing IPTG-inducible ARF (NARF cells) were used. MDM2 was immunoprecipitated from MG132 or IPTG-treated cells and tested for H3 methylation activity. ARF expression significantly reduced the level of methyltransferase activity associated with endogenous MDM2, both before and after DNA damage (Figure 11C and D). These results indicate that MDM2 interaction with histone methyltransferase is regulated by oncogenic stress through induction of ARF expression.

Discussion

The results described herein identify a novel mechanism of p53 transcriptional repression by MDM2. MDM2 recruitment of SUV39H1 and EHMT1 to p53 leads to the formation of trimeric complexes capable of promoting histone H3-K9

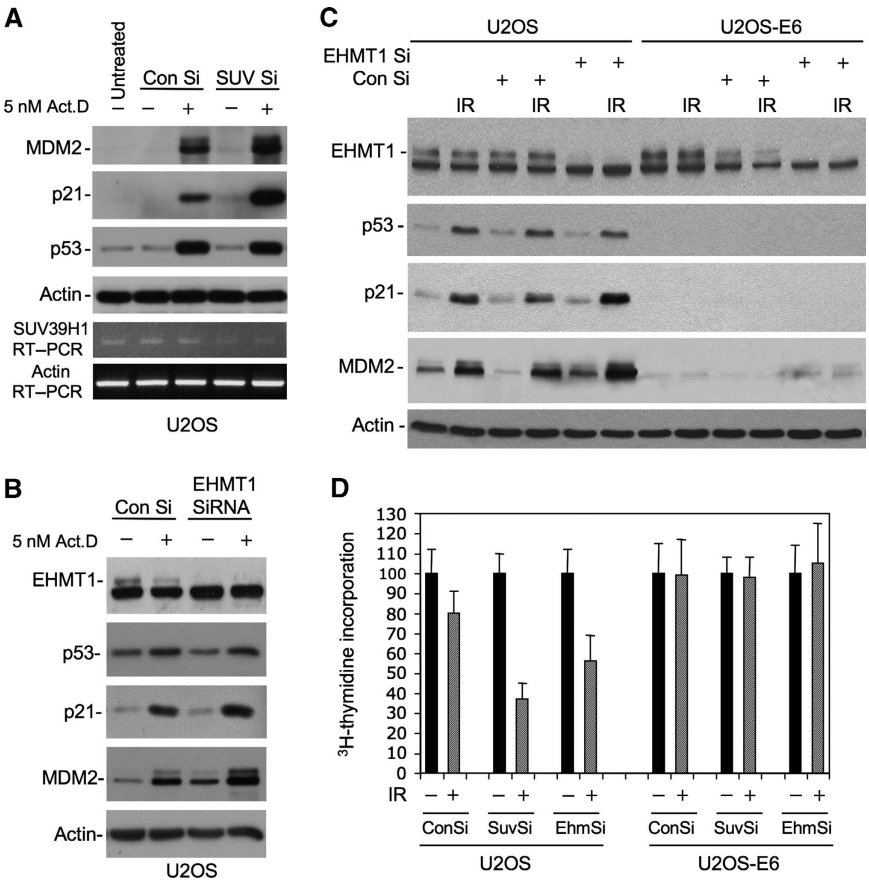

Figure 9 Knockdown of EHMT1 and SUV39H1 enhance p53 response to stress. (**A**) U2OS cells were transiently transfected with SUV39H1 siRNA for 48 h, treated with 5 nM actinomycin D for 18 h, and analysed for p53 response by western blot. (**B**) U2OS cells were transiently transfected with EHMT1 siRNA for 48 h, treated with 5 nM actinomycin D for 18 h, and analysed for p53 response by western blot. (**C**) U2OS cells were transiently transfected with EHMT1 siRNA for 48 h, treated with 10 IR for 4 h, and analysed for p53 response by western blot. U2OS-E6 cells were U2OS stably infected with HPV E6 retrovirus that promotes p53 degradation, thus behaving as functionally p53-null. (**D**) U2OS cells were transfected with SUV39H1 or EHMT1 siRNA for 48 h, treated with 4 Gy IR for 4 h, and labelled with ^{3}H-thymidine for 1 h. The rate of DNA synthesis was determined by scintillation counting of incorporated ^{3}H-thymidine.

methylation at p53 target promoters. We therefore propose that MDM2 can actively regulate chromatin conformation after its recruitment to DNA by p53. EHMT1 is known to promote H3-K9 mono- and di-methylation, whereas SUV39H1 may further modify the di-methylated K9 to a tri-methylated state (Rice *et al*, 2003). The two enzymes recruited by MDM2 may act sequentially or synergistically to stimulate tri-methylation of H3-K9 and achieve transcriptional repression. Furthermore, p53 can be methylated on K373 by EHMT1 and its homolog G9a. Although EHMT1 and G9a exhibit some intrinsic ability to methylate p53 in the absence of MDM2, MDM2 expression significantly stimulates p53 methylation through efficient recruitment of EHMT1 and G9a to p53. During the review of this manuscript, Huang *et al* (2010) reported that p53 is methylated on K373 by G9a and EHMT1. They also found that p53-mediated apoptosis was enhanced by knockdown of G9a and EHMT1, which is in agreement with our finding.

K373 acetylation by p300 and CBP results in stimulation of p53 transcriptional activity (Knights *et al*, 2006). Although the functional significance of p53 C-terminal acetylation is still a subject of debate, recent study indicated that acetylation may stimulate p53 tetramerization and thus enhance DNA binding (Li *et al*, 2006). Methylation of K373 by EHMT1–MDM2 complex is expected to interfere with acetylation and reduce p53 activity. Consistent with this notion, p53-373R mutant resistant to EHMT1 methylation is less sensitive to repression by MDM2–EHMT1 complex. Therefore, EHMT1 may regulate p53 by methylating both p53 K373 and histone H3-K9 at the target gene promoter. However, our results suggest that the fraction of endogenous p53 being methylated at K373 is likely to be small in the absence of ectopic MDM2 and EHMT1 expression. Therefore, the effect of EHMT1 may largely depend on methylation of H3 rather than p53.

It is noteworthy that K373 is located next to K372 which is modified by the SET9 methyltransferase (Chuikov *et al*, 2004). However, K373 methylation seems to be inhibitory, whereas K372 methylation stimulates p53 activity. How these opposing effects are achieved by modification of two adjacent lysine residues remains to be further investigated. An earlier study showed that methylation of K370 by Smyd2 inhibits p53 activity, possibly by interfering with K372 methylation by SET9 (Huang *et al*, 2006). Understanding whether K373 methylation by EHMT1 inhibits p53 activity by blocking K372 methylation requires further analyses.

Interestingly, MDM2 binding to EHMT1 only leads to p53 methylation on a single lysine residue. We did not observe methylation of MDM2 nor of MDMX in our assays. Furthermore, MDMX showed negligible binding to SUV39H1

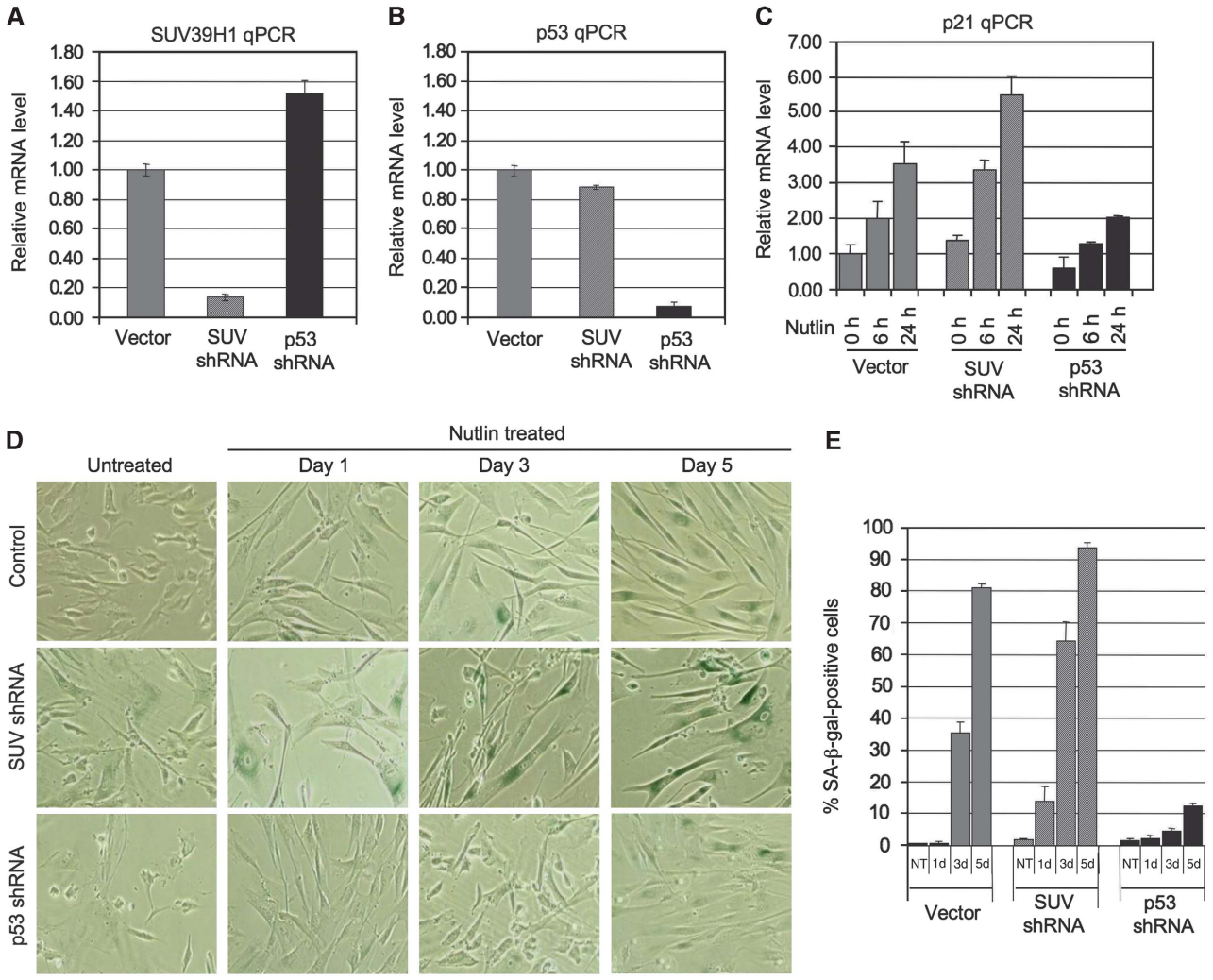

Figure 10 Knockdown of SUV39H1 enhances p53-induced senescence. (**A**, **B**) Human BJ foreskin fibroblasts were stably transduced with lentiviruses-expressing shRNA that targets SUV39H1 or p53. Knockdown of SUV39H1 or p53 was confirmed by RT–qPCR. (**C**) BJ fibroblasts with SUV39H1 and p53 knockdown were treated with Nutlin for 6 and 24 h and analysed for p21 expression by RT–qPCR. Data represent the mean (±s.d.) of three independent experiments. (**D**) BJ fibroblast cell lines were treated with 5 μM Nutlin for indicated periods and SA-β-Gal activity was revealed by staining. (**E**) Quantification of SA-β-Gal-positive cells in (**D**).

and EHMT1, and MDMX-containing complexes showed little associated methyltransferase activity. This specificity is consistent with the significant sequence divergence between the MDM2 and MDMX acidic domains, but is somewhat unexpected given that MDMX is an important regulator of p53 transcriptional activity mainly by sequestering it into a complex, whereas MDM2 can also promote p53 ubiquitination and degradation (Marine and Jochemsen, 2005).

Our results showed that increased MDM2 expression can promote H3-K9 methylation at the p21 promoter. But counterintuitively, DNA damage also increased the level of promoter K9 methylation while activating transcription. This is likely because of the significant increase in p53 and MDM2 levels during stress that increases the recruitment of SUV39H1 and EHMT1 to p53-binding sites through MDM2. Clearly, the increase in histone acetylation has a dominant effect in activating the promoters under such conditions, but the transcription efficiency is sub-optimal because of simultaneous rise of K9 methylation. Consistent with a function of MDM2–SUV39H1/EHMT1 complex acting as a negative feedback mechanism during p53 response, knockdown of SUV39H1 or EHMT1 can further enhance p53 transcriptional output.

Overall, our results support the notion that MDM2 functions by regulating both p53 degradation and p53 transcriptional activity. As MDM2 has a very short half-life because of self-ubiquitination, recruitment of histone and p53-methylating enzymes may serve to increase its p53 inhibitory function without relying on stable sequestration of p53. Furthermore, by recruiting repressors to p53, MDM2 converts p53 into an active transcriptional repressor, which is more effective than degradation alone. This is analogous to the example of pRb-E2F1 repressor complex. Not surprisingly, MDM2 interactions with SUV39H1 and EHMT1 are inhibited by the tumor suppressor ARF, which normally binds to the MDM2 acidic domain and causes p53 stabilization. The effect of ARF may be due to conformational change on MDM2 or by steric hindrance because of overlapping binding sites on the acidic domain. Therefore, ARF may function as an effective activator of p53 using two cooperating mechanisms: (1) inducing p53 accumulation and (2) functionally activating p53 by inhibiting MDM2 recruitment of methyltransferases

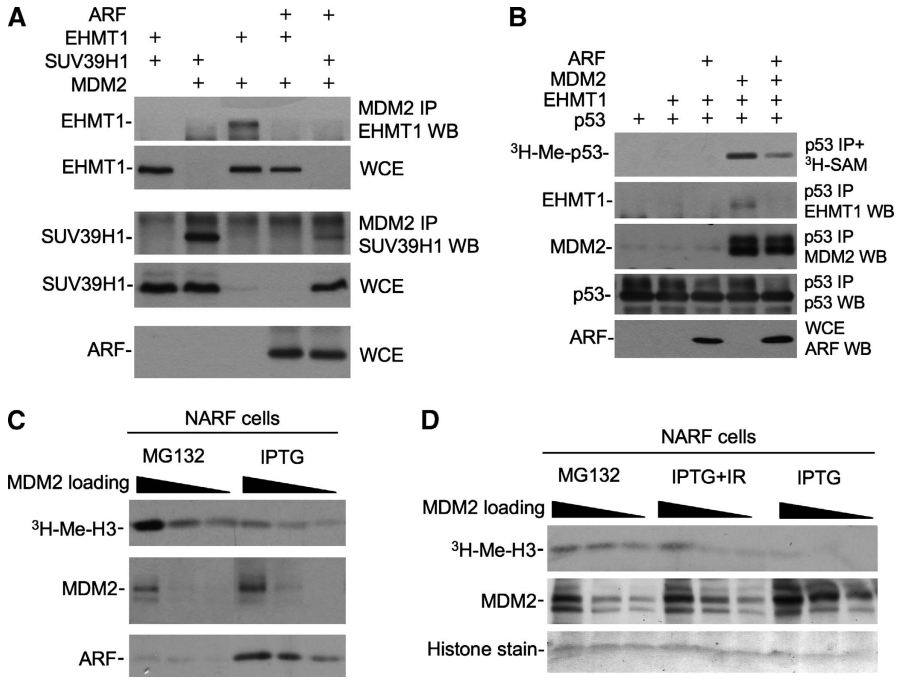

Figure 11 ARF inhibits MDM2 recruitment of EHMT1 and SUV39H1. (**A**) H1299 cells were transiently transfected with indicated plasmids. The effect of ARF expression on MDM2–EHMT1 and MDM2–SUV39H1 binding were determined by IP western blots. (**B**) H1299 cells were transfected with p53, MDM2, EHMT1, and ARF. P53 was immunoprecipitated and incubated with ^{3}H-SAM. P53 methylation was detected by autoradiography. The levels of MDM2 and EHMT1 coprecipitated with p53 were determined by western blot. (**C**, **D**) ARF expression in NARF cells was induced for 24 h with 0.1 mM IPTG, followed by irradiation with 10 Gy IR for 4 h. Endogenous MDM2 was immunoprecipitated and incubated with ^{3}H-SAM and core histones. Histone H3 methylation was detected by autoradiography. MDM2 stabilized by 4 h MG132 treatment was used as control. Samples were loaded at 100, 50, and 25% levels. MDM2 levels and ARF expression were confirmed by western blot.

and other transcription repressors (such as KAP1) (Wang *et al*, 2005).

Our overexpression and transient knockdown experiments demonstrated a function of endogenous SUV39H1 and EHMT1 in the regulation of p53-mediated transcription of target genes such as p21 and MDM2. However, SUV39H1-mediated histone H3-K9 methylation also has important functions in the establishment of permanently silenced heterochromatin regions. Formation of p53–MDM2–SUV39H1 complex on p53 target promoters could in principle initiate inheritable silencing of the promoters that cannot be immediately erased on removal of MDM2. However, our experiments using U2OS cells with tetracycline-reversible MDM2 expression indicated that long-term overexpression of MDM2 did not cause permanent change in p21 inducibility (unpublished observation). In fact, the level of H3-K9-Me3 level at p21 and PUMA promoters are much lower than constitutive heterochromatin. Therefore, our current data are most consistent with MDM2–SUV39H1 complex reversibly regulating p53 basal activity and stress response. Whether MDM2-mediated permanent repression can occur on certain p53 target genes, or has a function in establishing cell type-specific p53 transcriptional response remains a possibility to be further investigated.

Materials and methods

Cells and plasmids

MDM2/p53 double-null MEFs 174.1 were provided by Dr Gigi Lozano. NARFs (U2OS expressing IPTG-inducible p14ARF) were provided by Dr Dawn Quelle. H1299 (lung tumor, p53-null), U2OS (osteosarcoma, wt p53), SJSA (osteosarcoma, wt p53, MDM2 amplification), and 293T cells were maintained in DMEM medium with 10% fetal bovine serum. BJ human diploid fibroblasts and HEK293T human embryonic kidney cells were purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum. Stable BJ knockdown cell lines were generated after transduction and puromycin selection of lentiviruses-expressing shRNA against p53 (pSIF, System Biosciences), SUV39H1 (pGIPZ, OpenBiosystems cat. no. RHS4430-98843211) or control vector containing a scrambled shRNA (pGIPZ, OpenBiosystems cat. no. RHS4346). U2OS cell line with stably transfected tet-off MDM2 were described earlier (Pan and Chen, 2003). P53 point mutants were generated by site-directed mutagenesis using the QuickChange kit (Stratagene). All p53, MDM2, and MDMX constructs used in this study were human cDNA clones. MDM2 inhibitor Nutlin (Cayman) was used at 5–10 μM. SUV39H1 and EHMT1 knockdown (Figure 9) was accomplished by transfection of 100 nM Smartpool siRNAs (Dharmacon) using Lipofectamine for 56 h. A second set of custom-designed siRNA (SUV39H1: GGTGAAATGGCGTGGATAT. EHMT1: GC ACAGCCCTTGAGGACTA) were used for confirmation experiment (Supplementary Figure S4).

Protein analysis

To detect proteins by western blot, cells were lysed in lysis buffer (50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF) and centrifuged for 5 min at 10 000 *g*. Cell lysate (10–50 μg protein) was fractionated by SDS–PAGE and transferred to Immobilon P filters (Millipore). The filter was blocked for 1 h with phosphate-buffered saline containing 5% non-fat dry milk, 0.1% Tween-20. The following antibodies were used: 3G9 and 5B10 for human MDM2 western blot and IP (Chen *et al*, 1993); rabbit polyclonal MDM2 antibody for western blot of MDM2 deletion mutants; Pab1801 for p53 IP; DO-1 (Pharmingen) for p53 western blot; 8C6 antibody for MDMX western blot and IP (Li *et al*, 2002). The filter was developed using ECL-plus reagent (Amersham).

SUV39H1 antibody was purchased from Millipore. EHMT1 antibody was purchased from Bethyl Laboratories. P53 methylation-specific rabbit antibody was raised against peptide HLKSK(monomethyl-K)GQSTSC (Biosynthesis Inc.) and affinity purified using methylated and unmethylated p53 peptide columns, followed by further depletion using His6-p53 purified from *Escherichia coli*. P53-methylation analysis was carried out by immunoprecipitation of 0.5–2 mg cell extract using Pab1801 antibody cross-linked to protein A beads followed by western blot using the methylation-specific antibody.

In vitro methylation assay

To detect MDM2-associated methyltransferase activity, MDM2 was immunoprecipitated from a 10-cm plate culture (transfected or drug treated) using 5B10 or 4B2 monoclonal antibodies, washed with SNNTE buffer (50 mM Tris–HCl pH 7.4, 500 mM NaCl, 5 mM EDTA, 0.1% NP40, 5% sucrose), and washed with methylation buffer (50 mM Tris–HCl pH 8.5, 5 mM $MgCl_2$, 4 mM DTT). The beads ($\sim$20 μl bed volume) were mixed with 15 μl methylation buffer, 1 μg GST-histone H3, 5 μg core histones (Sigma), 0.1 μg His6-p53, 2 μCi ^{3}H-SAM (15 Ci/mmol) and incubated for 2 h at 30°C with mixing at 600 r.p.m. The sample was boiled in Laemmli sample buffer, fractionated by SDS–PAGE, transferred to nylon membrane, sprayed with EN3HANCE, and exposed against film for 8–48 h at −80°C. The membrane was probed for MDM2 level by western blot. To detect p53 methylation, p53 was cotransfected with MDM2 and EHMT1 into H1299 cells, immunoprecipitated using Pab1801 antibody, and incubated with ^{3}H-SAM.

Chromatin immunoprecipitation

ChIP assay (Figure 4) was performed using standard procedure. H1299 cells were transfected with 1 μg p53, 5 μg SUV39H1, 5 μg EHMT1, 5 μg MDM2 plasmids in 10 cm plates for 24 h. Cross-linked chromatin were sonicated and immunoprecipitated using anti-Myc antibody. Samples were subjected to PCR analysis using forward and reverse primers for the p53-binding sites in the MDM2 promoter (5′-CGGGAGTTCAGGGTAAAGGT and 5′-CCTTTTACTG CAGTTTCG) and p21 promoter (5′-TGGCTCTGATTGGCTTTCTG and 5′-TTCAGAGTAAGAGGCTAAGG). The following antibodies were used for H3-K9 ChIP analyses (Figure 8): GAH-6204 (Qiagen) for K9-Me3, Aab7312 (Abcam) for K9-Me2 and 06-942 (Upstate) for K9-Ac. Quantitative PCR was performed using p21 promoter primers P21B2F (TCTAGGTGCTCCAGGTGCTT) and P21B2R (TCTG GCAGGCAAGGATTTAC).

Luciferase reporter assay

MDM2/p53 double-null MEF (174.1 line) were cultured in 24-well plates (50 000/well) and transfected with a mixture containing 20 ng p53-responsive BP100-luciferase reporter, 5 ng CMV-lacZ, 0.1 ng p53, 3 ng MDM2, and 10 ng EHMT1 or 30 ng SUV39H1 plasmids. Transfection was achieved using Lipofectamine PLUS reagents (Invitrogen) and cells were analysed for luciferase and β-galactosidase expression after 24 h. The ratio of luciferase/β-galactosidase activity was used as indicators of transcriptional activity.

Senescence assay

Stable BJ knockdown lines were seeded in six-well plates on round glass coverslips (120 000 cells/well) and treated with 5 μM Nutlin for 1, 3, and 5 days. Senescence was assessed using Senescence β-Galactosidase Staining Kit (Cell Signaling). Images were captured using an inverted fluorescence microscope (Olympus IMT 200) equipped with ×20/0.4 Plan Phase contrast LWD objective, DAPI filter set, PixeLINK PL-A662 digital camera, and PixeLINK Capture SE software version 3.1. The percentage of SA-β-galactosidase-positive cells was estimated by calculating the number of blue-stained cells per 1000 DAPI-labelled nuclei from three parallel experimental replicas per cell line, using ImageJ software.

Mass spectrometry

Matrix-assisted laser desorption ionization mass spectrometry was performed with a tandem time-of-flight instrument (4700, Applied Biosystems, Framingham, MA). After deposition with α-cyano-4-hydroxycinnamic acid matrix, peptide analytes were analysed in positive ion mode for both MS and MS/MS sequence analysis to localize the sites of methylation. Spectra were searched against human protein entries in the SwissProt database with Mascot (http://www.matrixscience.com) and then manually interpreted for verification.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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