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E6 Protein Expressed by High-Risk HPV Activates Super-Enhancers of the *EGFR* and *c-MET* Oncogenes by Destabilizing the Histone Demethylase KDM5C

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Abstract

The high-risk (HR) human papillomaviruses (HPV) are causative agents of anogenital tract dysplasia and cancers and a fraction of head and neck cancers. The HR HPV E6 oncoprotein possesses canonical oncogenic functions, such as p53 degradation and telomerase activation. It is also capable of stimulating expression of several oncogenes, but the molecular mechanism underlying these events is poorly understood. Here, we provide evidence that HPV16 E6 physically interacts with histone H3K4 demethylase KDM5C, resulting in its degradation in an E3 ligase E6AP- and proteasome-dependent manner. Moreover, we found that HPV16-positive cancer cell lines exhibited lower KDM5C protein levels than HPV-negative cancer cell lines. Restoration of KDM5C significantly suppressed the tumorigenicity of CaSki cells, an HPV16-positive cervical cancer cell line. Whole genome ChIP-seq and RNA-seq results revealed that CaSki cells contained super-enhancers in the proto-oncogenes *EGFR* and *c-MET*. Ectopic KDM5C dampened these super-enhancers and reduced the expression of proto-oncogenes. This effect was likely mediated by modulating H3K4me3/H3K4me1 dynamics and decreasing bidirectional enhancer RNA transcription. Depletion of KDM5C or HPV16 E6 expression activated these two super-enhancers. These results illuminate a pivotal relationship between the oncogenic E6 proteins expressed by HR HPV isotypes and epigenetic activation of super-enhancers in the genome that drive expression of key oncogenes like *EGFR* and *c-MET*.

Significance: This study suggests a novel explanation for why infections with certain HPV isotypes are associated with elevated cancer risk by identifying an epigenetic mechanism through which E6 proteins expressed by those isotypes can drive expression of key oncogenes. *Cancer Res; 78(6); 1418–30. ©2018 AACR.*

Introduction

The human papillomaviruses (HPV) are nonenveloped DNA viruses that infect human epithelia tissues. Infection by the high-risk (HR) types, HPV-16 and HPV-18, are the major causes of anogenital carcinomas in women and men, as well as a fraction of head and neck cancer (1, 2). The early proteins E6 and E7 of the HR HPVs are oncoproteins. Constitutive expression at high levels can immortalize primary human epithelial cells and induce tumors in animal models (3). The HR HPV E7 protein interacts and destabilizes retinoblastoma tumor suppressor family (RB1 and RB2), rendering the host cells to bypass G_0–G_1 to S phase controls (4). In concert with the dysregulated cell growth, the HR HPV E6 protein forms a complex with the ubiquitin ligase E6AP and the tumor suppressor p53, resulting in its destabilization. The HR HPV E6 also interacts and destabilizes a number of other proteins, such as MGMT, BAK, hADA3, TIP60 (5, 6), BRCA1 (7), and caspase-8 (8). These interactions broadly affect host cell signal transduction, chromatin remodeling, genome stability, and apoptosis, suggesting that HPV-associated carcinogenesis involves a coordinated targeting of multiple pathways. Certainly, additional targets and pathways remain to be discovered.

As a noncanonical function, HR HPV E6 plays an important role in regulating certain oncogene expression. For example, HR HPV E6 increases the epidermal growth factor receptor (*EGFR*) expression (9–11). Upregulation of *EGFR* has been reported in cervical cancers; it contributes to cancer cell proliferation, migration, and invasion. Another highly expressed proto-oncogene in cervical cancer is *c-MET* (12), which is known to promote the occurrence and development of cervical cancer and has prognostic value (13). Given the fact that cervical carcinomas are caused by persistent HR HPVs infection, elevated proto-oncogene expression is also possibly a consequence of the HR HPVs infection (14). However, the molecular mechanisms underlying the upregulation of these two proto-oncogenes are unknown.

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Oncoproteins may utilize transcription factors to maintain the general transcriptional machinery and sustain the oncogenic state. Although HR HPV16 E6 activates NF-κB (15), a complex that regulates transcription to change the cellular environment, it cannot explain the elevated proto-oncogene expression in cervical cancers. Another emerging role in proto-oncogene activation is the abnormal epigenetic modifications. DNA methylation, histone modifications, nucleosome remodeling, and non-coding RNA-mediated targeting regulate many biological processes that are fundamental to the genesis of cancer (16). Because modifications to DNA and histones are dynamically laid down and removed by chromatin-modifying enzymes in a highly regulated manner, these enzymes are frequently mutated or dysregulated in various types of cancer (17). Histone lysine methylation is one of many key epigenetic modifications. In general, histone H3 lysine 4 (H3K4), H3K36, and H3K79 methylation are gene-activation marks, whereas H3K9, H3K27, and H4K20 methylation are gene-repression modifications. Histone lysine methylation is generated or erased by a number of histone methyltransferases (HMT) or removed by histone demethylases. For example, H3K4 tri-methylation (H3K4me3) is mediated by MLL1, MLL2, MLL3, MLL4, Ash2L, SETD1A, SETD1B, WDR5, and RBBP5, whereas it is removed by histone demethylase KDM5A, KDM5B, and KDM5C (18–20). Previous studies have shown that HR HPV E6 interacts with both histone methyltransferases (CARM1, SET7, and PRMT1; ref. 21) and acetyltransferases (p300, hADA3, and Tip60; ref. 22); but it is not always clear how these interactions induce changes in chromatin structures or alter gene expression in cervical cancer. Importantly, recent study suggested that, in cancer cells, enhancers that normally control the signal-dependent expression of growth-related genes are frequently dysregulated. Moreover, the generation and activation of super-enhancers can be a persistent regulatory element that drives the uncontrolled proliferation (23). A number of super-enhancers have been identified in various types of cancer, but it has not been reported in cervical carcinomas.

Here, we demonstrate that HPV16 E6, but not the non-oncogenic HPV6b E6, interacts with KDM5C, a tumor suppressor (24) and histone H3K4me2/3-specific demethylase, and, in the presence of E6AP, mediates its polyubiquitination at K1479, leading to degradation in a proteasome-dependent manner. Consistently, ectopic expression of HPV16 E6 leads to a moderate H3K4 methylation alteration. We have also found that HPV16-positive cervical cancer cell lines exhibit lower KDM5C protein levels than HPV-negative cancer cell lines. Conversely, restoration of KDM5C in CaSki cells, an HPV16-containing cervical cancer cell line, significantly suppresses its growth and invasion *in vitro* and tumorigenesis in nude mice. By using systematic molecular analyses, we show that CaSki cells contain both *EGFR* and *c-MET* super-enhancers. Ectopic KDM5C expression reduces their activity while KDM5C depletion increases their activities. Dramatically, the presence of HPV16 E6 is sufficient to upregulate the *EGFR* and *c-MET* super-enhancers, further elevating the expression of these two proto-oncogenes. We therefore propose that HR HPV E6 activates cervical cancer super-enhancers and promotes tumorigenesis by targeting tumor suppressor KDM5C.

Materials and Methods

Vectors and plasmids

HPV16 E6, the N-terminal (1–43) and the C-terminal portion (44–151) of HPV16 E6 were cloned into pEGFP-C1 for mammalian cell expression. HPV16 E6 mutation constructs were generated similarly. HPV16 E6 Y79N has a single substitution at tyrosine at residue 79. HPV16 E6 F2V/P5R has double substitutions. HPV16 E6 Δ149–151 mutation has deletion of amino acids 149 to 151. In HPV16 E6 (1–43) and 16 E6 (44–151), the amino acids 1–43 and 44–151 are preserved, respectively. The pHAGE vector carries a puromycin-resistance cassette, conferring the transfected cells resistance to puromycin at varying concentrations, hence enabling us to select and generate stable cells. The KDM5C K1479R mutation was constructed by overlapping extension during polymerase chain reaction-PCR from the pHAGE-KDM5C plasmid. For bacterial expression of fusion to the maltose binding protein (MBP), HPV16 E6 or mutations were each cloned into pMAL-c2x vector. All the constructs were validated by DNA sequencing.

Cell culture and reagents

CaSki, C33A, SiHa, and HeLa cell lines were purchased from the ATCC between 2012 and 2014. HEK293T, HCT116, and U2OS cell lines were obtained from Institute of Cellular Resources, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences in 2011 and 2015. All cell lines involved in our experiments were reauthenticated by short tandem repeat analysis every 6 months after resuscitation in our laboratory and routinely tested to verify the absence of mycoplasma with LookOut Mycoplasma PCR Detection Kit (Sigma). CaSki and C33A cell lines were additionally tested for the presence of HPV16 by PCR before the initiation of this study. The cells revived from frozen stocks were used within 10 to 20 passages or for no longer than 2 months in total for any experiment.

HCT116, HEK293T, C33A, and SiHa cells were cultured in Dulbecco's Modified Eagle's Medium-DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS, Gibco). CaSki, U2OS cells were maintained in Roswell Park Memorial Institute-RPMI 1640 medium (HyClone) with 10% FBS (Gibco). Stable vector (CaSki-pHAGE)-containing cells and KDM5C expressing CaSki cells (CaSki-KDM5C) were generated by lentivirus transduction and selected with puromycin. Briefly, lentiviruses were generated in HEK293T cells transfected with helper plasmids pMD2.G and psPAX2, together with vector or KDM5C-expressing vector using Lipofectamine 2000 (Invitrogen, 11668). CaSki cells were then infected with lentivirus. Stably transduced cells were selected in RPMI-1640 (HyClone) supplemented with 10% FBS (Gibco) and 2 μg/mL puromycin (Cayman Chemicals, 13884). Stable C33A cell lines expressing vector (C33A-EGFP) or EGFP-HPV16 E6 (C33A-16E6) were established as described above. C33A cells were subsequently infected with lentivirus and maintained in DMEM (HyClone) medium with 4 μg/mL puromycin. These cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

The CaSki KDM5C knockout (CaSki-KDM5C KO) cell line was generated as described in Sanjana and colleagues (25). Lentivirus carrying the guide RNA (gRNA) of KDM5C was produced as mentioned above and used to infect CaSki cells. The lentiviral based lentiCRISPRv2-KDM5C plasmid was kindly provided by Yang Shi (Harvard University). Stable CaSki-KDM5C KO cells were selected in appropriate complete medium supplemented with 2 μg/mL puromycin. Single clones were established in the absence of puromycin and knockout efficiency were periodically confirmed by Western blot. gRNA sequence used is KDM5C exon 1: 5′-ACGATTTCCTACCGCCACC-3′.

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RNA extraction and qRT-PCR

Total RNA was obtained from treated cell lines using TRIzol (Invitrogen, 15596018) according to the manufacturer's instructions. For cDNA preparation, 1 μg of total RNA was reverse transcribed for RT-PCR using All-in-One cDNA synthesis SuperMix (Biotool, B24403). The obtained cDNA was amplified by PCR for 16 E6 expression analysis. The PCR primers were designed with Primer Premier 5.0 software and GAPDH was used as a reference gene. qPCR was performed on CFX Connect Real Time PCR Detection System (Bio-Rad) using SYBR Green Real time PCR Master Mix (Biotool, B21802). GAPDH amplification products were used as a control. All reactions were carried out in triplicates. Data were analyzed by the $2^{-\Delta\Delta CT}$ method. Primer sequences are detailed in Supplementary Table S1 in the Supporting information.

RNA-seq and analysis

Total RNA was extracted from CaSki-pHAGE and CaSki-KDM5C cells using TRIzol (Invitrogen, 15596018). Purity of RNA samples was determined with NanoPhotometer Spectrophotometer (Implen) and concentration was quantified in a Qubit 2.0 fluorometer (Life Technologies). Sequencing libraries were constructed using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, E7420) and library quality was validated on Bioanalyzer 2100 (Agilent Technologies). Clustered sequences were generated using TruSeq SE Cluster Kit v3-cBot-HS (Illumina) followed by sequencing on HiSeq 2500 (Illumina). Generated reads were mapped to human genome (hg38) database using TopHat v2.0.9. Gene expression levels and differential expression were quantified with Cuffdiff v2.1.1. Threshold for differential expression was set at FPKM $\geq$ 1 and P value < 0.05.

Chromatin immunoprecipitation-PCR and ChIP-Seq

See details in Supplementary section.

Primer sequences used in this assay were listed in Supplementary Table S2.

Xenograft assay

Five-week-old female BALB/c nude mice were acquired from Beijing HFK Bioscience (Beijing, China) and housed under specific pathogen-free conditions in Animal Experiment Center-Animal Biosafety Level-III Laboratory of Wuhan University. The animals were fed with standard laboratory mice diet and water *ad libitum*. At 6th week, mice were randomly divided into 2 groups and injected with 100 μL of stable CaSki-pHAGE or CaSki-KDM5C cells suspended in Matrigel Basement Membrane Matrix (Corning, 356234) at a density of 5×10^6 cells into the left dorsal flank subcutaneously after alcohol sterilization of injection site skin surface. Tumor size and body weight of each mice were obtained once weekly using digital vernier caliper and electronic scale, respectively. Tumor volume was calculated with the formula: tumor volume (mm^3) =length (mm) $\times$ width (mm^2) $\times$ 0.5. Mice were sacrificed by cervical dislocation and tumors were excised on 6th week after injection. All animal experiments performed were approved by and conform to the guidelines of Animal Research Ethics Board of Wuhan University.

The xenograft of CaSki KDM5C knockout cells (CaSki-KDM5C KO) was performed as mentioned above, and CaSki cells were used as control group.

Additional information is presented in Supplementary Methods.

Sequencing data have been deposited in the Gene Expression Omnibus(GEO) repository under accession code GSE101565.

Results

HPV16 E6 interacts with histone demethylase KDM5C

Previous studies showed that histone demethylase KDM5C (also called SMCX or JARID1C) and histone acetyltransferase TIP60 each forms a complex with HPV E2 protein to repress the viral LCR promoter (26, 27). Conversely, the oncogenic and non-oncogenic HPV E6 proteins interact with TIP60 and destabilize it in a proteasome-dependent manner to de-repress the LCR promoter (6). It is not known whether KDM5C could also be targeted by HPV E6 in a similar manner. Because HPV-induced malignancies are largely the result of protein–protein interactions involving viral oncoproteins, we began our studies by examining a possible interaction between HPV16 E6 and KDM5C.

First, we expressed EGFP-16 E6 fusion protein in HPV-negative human cervical cancer cell line C33A. Coimmunoprecipitation clearly showed that HPV16 E6 bound to endogenous KDM5C (Fig. 1A). We also coexpressed EGFP-16 E6 and KDM5C in HCT116 cells (epithelial cells derived from colorectal carcinoma) and performed reciprocal coimmunoprecipitations. The result also showed that KDM5C specifically bound to HPV16 E6 (Supplementary Fig. S1). To rule out the possibility that EGFP itself interacts with KDM5C, we performed coimmunoprecipitation by coexpressing KDM5C and EGFP-16 E6 or EGFP in HCT116 cells. The results showed that KDM5C was coimmunoprecipitated only in the presence of EGFP-16 E6 but not EGFP (Fig. 1B). To confirm that HPV16 E6 interacted directly with KDM5C, we further constructed and purified the MBP-16 E6 fusion protein from *E. coli*. We generated the ectopically and stably expressed KDM5C in CaSki cells (CaSki-KDM5C). The purified MBP-16 E6 or MBP protein was then incubated with CaSki-KDM5C cell lysates and pulldown assays were performed. As shown in Fig. 1C, purified MBP-16 E6 fusion protein but not MBP clearly interacted with KDM5C.

HPV E6 protein interacts with cellular proteins via various domains. For example, the S/TXV motif at HR HPV E6 C-terminus interacts with PDZ domain–containing proteins, whereas the N-terminus with p53. To delineate the HPV16 E6 domain, which interacts with KDM5C, we constructed a series of MBP-fused HPV16 E6 mutant forms and performed pulldown assays using lysates of CaSki-KDM5C (Fig. 1D). As shown in Fig. 1E, F2V/P5R, a double point mutation of HPV16 E6 protein, which is unable to bind p53, interacted with KDM5C, as the wild-type protein did (lane 3). The Y79N mutation, which abolishes the interaction between HPV16 E6 and E3 ligase E6AP (24), did not influence E6-KDM5C interaction (lane 5). Notably, the deletion of S/TXV motif at E6 C-terminus slightly attenuated the interaction (lane 4), whereas the deletion of amino acid residues 44–151 completely abolished the binding (lane 7). In fact, the expression of residues 44–151 retained the full binding activity (lane 6). These results indicate that HPV16 E6 interacts with KDM5C via the central and C-terminal portions of the protein, different from those that interact with the p53 and PDZ family of proteins. Although KDM5C does not contain PDZ and LXXLL motifs, a number of HR HPV E6-binding cellular proteins, such as p53, Bak, p300/CBP, hADA3, NFX1, Gps2, FADD, TIP60, and procaspase-8, do not have these motifs either (8).

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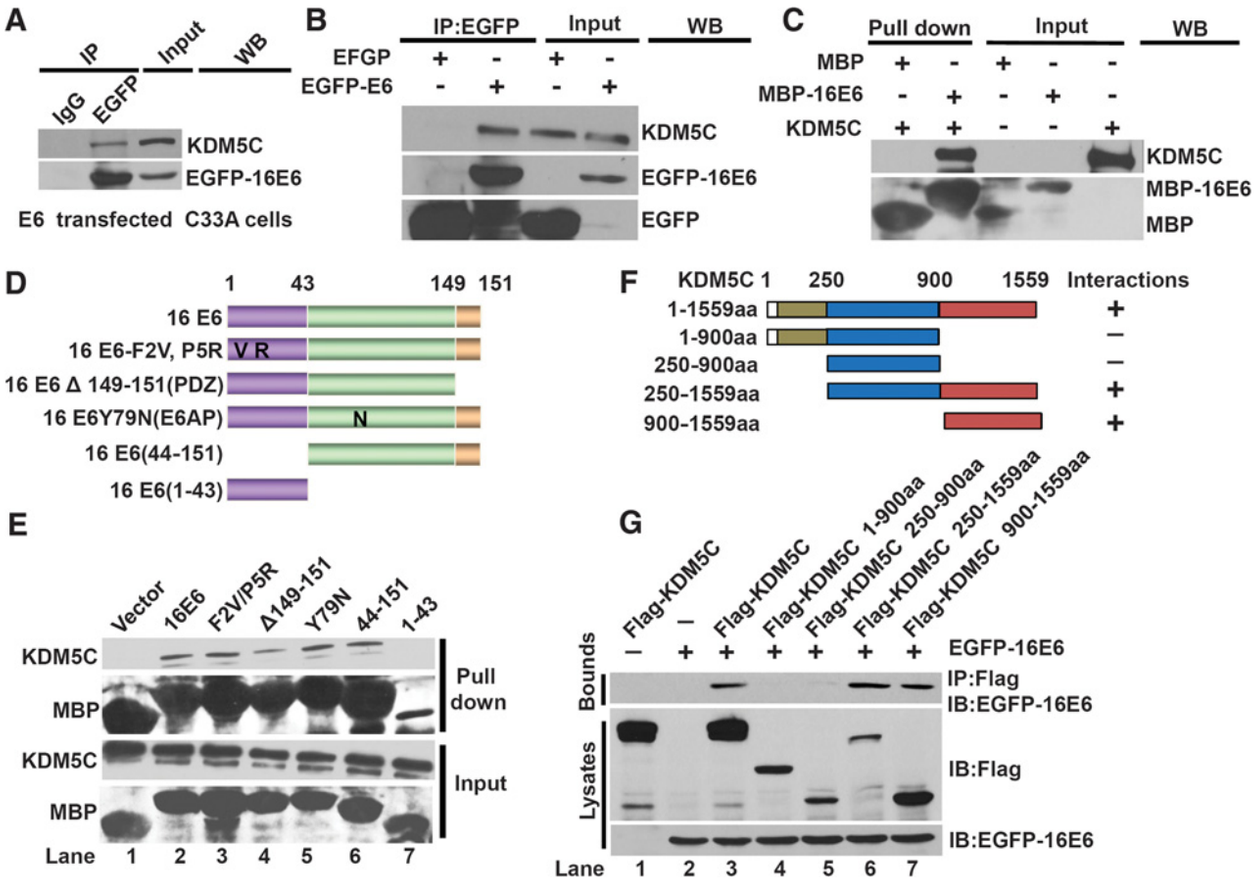

Figure 1.

Identification of interactions between HPV16 E6 and KDM5C. **A,** Coimmunoprecipitation of HPV16 E6 and KDM5C in cervical C33A cells overexpressing E6 protein. **B,** Coimmunoprecipitation by coexpressing KDM5C and EGFP-16E6 or EGFP, respectively, in HCT116 cells to confirm KDM5C interacts with HPV16 E6. **C,** Interaction between HPV16 E6 and KDM5C in MBP pulldown assay performed with purified MBP-16E6 and CaSki-KDM5C cell lysates. **D,** Schematic representation of HPV16 E6 mutation constructs. Descriptions of each construct are elaborated in Materials and Methods. **E,** Determining interactions between KDM5C and mutated HPV16 E6. MBP pulldown assay performed with purified MBP fused HPV16 E6 wild-type or mutant forms of the 16E6 protein and CaSki-KDM5C cell lysates. **F,** Schematic representation of KDM5C truncation constructs. **G,** Each KDM5C truncation construct was cloned into the pHAGE vector harboring Flag-tag. Immunoprecipitation assays were carried out to determine interaction between each truncation construct with HPV 16E6; KDM5C C-terminal region is required for interaction with HPV 16E6.

We also constructed KDM5C truncation mutations and delineated the domain of KDM5C responsible for 16E6 binding. Flag-tagged wild-type and four mutant forms were each cotransfected with the GFP-16 E6 vector into HCT116 cells. The pulldown result showed that C-terminal region of KDM5C is required for a stable interaction with E6 (Fig. 1F and G).

HPV16 E6 destabilizes the KDM5C

HR HPV E6 disrupts normal cellular metabolism by dysregulating gene expression, inactivating protein functions, or promoting the target protein degradation. We therefore wanted to know if HPV16 E6 could alter the KMD5C protein level. We examined the KDM5C protein level by immunoblots in U2OS cells (epithelial cells derived from osteosarcoma). The endogenous wild-type p53 was used as positive control for E6-target degradation. As shown in Fig. 2A and B, in the presence of expression vector of EGFP-HPV16 E6, but not the EGFP vector, the p53 protein level was significantly reduced. Notably, the endogenous KDM5C, but not its family members KDM5A and KDM5B, exhibited a decreased protein level. Similar results were obtained in HCT116 cells (Supplementary Fig. S2A and S2B).

To analyze systemically the effect of HPV 16E6 motifs on KDM5C stability, we expressed wild-type HPV 16E6 or various HPV 16E6 mutations and detected the KDM5C protein level. As shown in Fig. 2C, full-length HPV 16E6 expression eliminated most of the KDM5C and p53, whereas the E6AP binding–defective 16E6 mutation Y79N and 16E6 N-terminus (1–43) failed to destabilize KDM5C or p53 (lanes 5 and 7). In contrast, the 16E6 mutant F2V/P5R, a previously reported p53 binding–defective mutant protein, was able to efficiently destabilize the KDM5C, but not p53 (lane 3). Interestingly, the 16E6 (44–151) led to a significantly reduced KDM5C but not p53 protein (lane 6). These results were consistent with the pulldown assays as described previously and indicate that an interaction of HPV16 E6 and KDM5C is important for KDM5C destabilization. We also expressed GFP-E6 in HCT116 cells and performed indirect immunofluorescence assay. As shown in Fig. 2D, the KDM5C protein (red) was significantly decreased in cells expressing EGFP-HPV16

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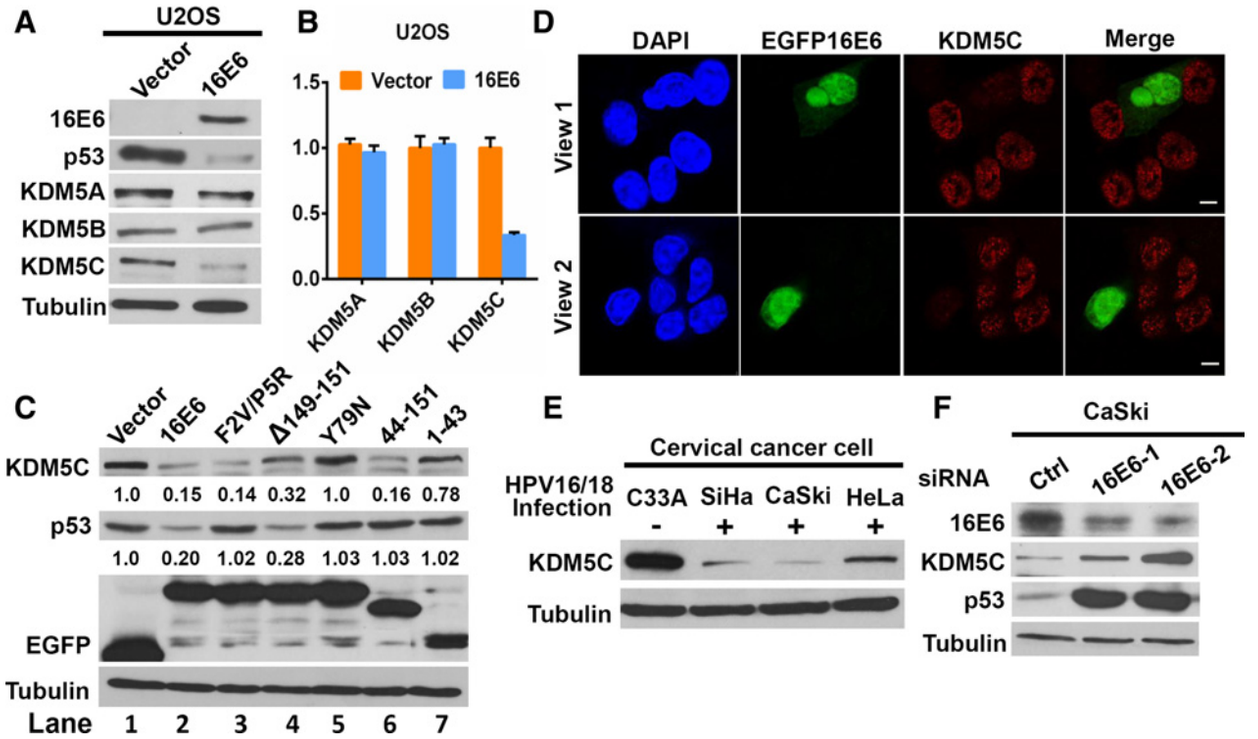

Figure 2.

HPV16 E6 destabilizes KDM5C. **A,** U2OS cells transfected with vector or HPV16 E6. Immunoblot analyses were performed and expression changes were detected using antibodies against p53, KDM5A, KDM5B, and KDM5C. **B,** Protein expression of KDM5A, KDM5B, and KDM5C in U2OS cells overexpressing HPV16 E6 or vector was quantified based on blot intensities of three independent experiments in **A**. **C,** HPV16 E6 mutation constructs were cloned into pEGFP-C1 and transfected into HCT116 cells. Protein level changes of KDM5C and p53 were detected by immunoblot. **D,** Indirect immunofluorescence microscopy was performed in HCT116 cells overexpressing HPV16 E6. EGFP16 E6 (green), KDM5C (red), and DAPI (blue). Scale bars, 5 μm. **E,** KDM5C expression in four cervical cancer cells was examined by immunoblots. **F,** KDM5C and p53 protein levels after siRNA targeting HPV16 E6 were introduced in CaSki cells.

E6 (green) relative to cells without HPV16 E6. Quantification of the KDM5C mRNA in HCT116 cells by qPCR showed it remained unchanged in the presence or absence of ectopic HPV16 E6 (Supplementary Fig. S2C).

We then extended our investigation into cervical cancer cell lines that are negative or positive for HR HPVs. As shown in Fig. 2E, the KDM5C protein level in HPV-negative C33A cells was clearly higher than that in two HPV16-positive cancer cell lines, CaSki and SiHa, as well as one HPV18-positive cancer cell line, HeLa. These results are in agreement with previous observations with ectopic expression of HPV16 E6 in HPV-negative cell lines (Fig. 2A, B, and D). To rule out the possibility that factors other than HPV16 E6 may have led to the varied KDM5C levels in cervical cancer cell lines, we transiently knocked down HPV16 E6 in CaSki cells. Depletion of E6 increased both p53 and KDM5C protein levels, reinforcing the idea that HPV16 E6 destabilizes KDM5C (Fig. 2F).

HPV16 E6 promotes the KDM5C degradation via the ubiquitin pathway

One of the most characterized properties of HPV16 E6 is its ability to induce the tumor suppressor p53 degradation via the ubiquitin pathway. Because HPV16 E6 destabilizes KDM5C, which is another tumor suppressor identified in the recent years (28), we explored if KDM5C was also degraded by a similar mechanism.

First, we treated CaSki and C33A cells with the proteasome inhibitor MG-132. As shown in Fig. 3A, the KDM5C protein level increased in the HPV-positive CaSki cells but not in HPV-negative-C33A cells. The same result was observed when U2OS cells were transfected with HPV16 E6 and exposed to MG-132. The KDM5C protein level was comparable with that in cells not expressing HPV16 E6 (Fig. 3B). These results indicate that HPV16 E6 destabilizes KDM5C in a proteasome-dependent pathway. We next asked that if E6AP plays the same role in the KDM5C degradation as it does in destabilizing p53. Indeed, upon knocking down E6AP, both p53 and KDM5C protein levels increased (Fig. 3C), demonstrating that KDM5C is degraded by HPV16 E6/E6AP-mediated proteasome activity, as is p53.

To validate our observation, we expressed EGFP, EGFP-HPV16 E6, or E6 truncation mutation forms along with HA-ubiquitin, KDM5C in HCT116 cells. IP was performed with anti-KDM5C and ubiquitin signal was detected with anti-HA in the presence of MG-132. The presence of EGFP-HPV16 E6 and truncation mutation E6 (44–151), but not the EGFP or the truncation mutation E6 (1–43), clearly showed polyubiquitination of KDM5C (Fig. 3D). Thus, the E6 N-terminal portion is dispensable for KDM5C polyubiquitination. To obtain further insights into the mechanism of the KDM5C ubiquitination, the IP products were

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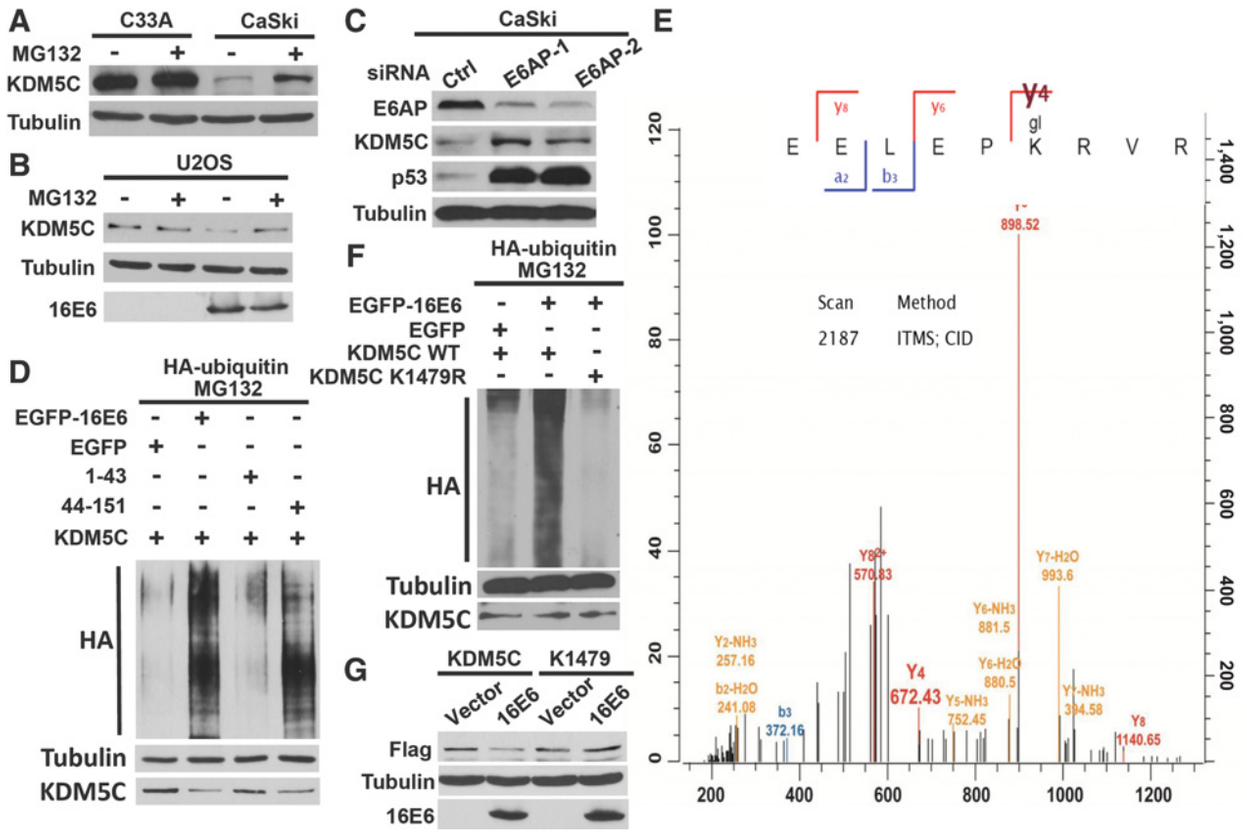

Figure 3.

HPV16 E6 promotes KDM5C degradation via the ubiquitin proteasome pathway. **A,** MG132-treated CaSki cells show increased KDM5C protein level as compared with C33A cells. **B,** HPV16 E6 was overexpressed in U2OS cells. KDM5C expression in cells with or without MG132 treatment was analyzed by immunoblots. **C,** E3 ubiquitin ligase E6AP was knocked down by siRNA in CaSki cells; its effect on p53 and KDM5C protein level was evaluated by immunoblots. **D,** Polyubiquitination assays were performed in HCT116 cells cotransfected with plasmids expressing HA-ubiquitin, KDM5C, HPV16 E6, HPV16 E6 mutants, or vector. MG132 was added to 10 μmol/L for 4 hours prior to harvest. **E,** Lysates of ubiquitination assay were subjected to mass spectrometry analysis. MS/MS spectrum indicates KDM5C ubiquitination on lysine K1479. **F,** KDM5C mutation K1479R in which arginine was substituted for lysine is defective in HPV16E6-mediated polyubiquitination. **G,** HPV16 E6 degrades wild-type KDM5C but not K1479R mutant. HPV16 E6 (2 μg) was cotransfected with 0.1 μg Flag-KDM5C wt or K1479R mutant in HCT116 cells and the KDM5C protein level was detected using Flag tag antibody.

subjected to mass spectrometry analysis. The result revealed that the lysine residue at 1479 of KDM5C was ubiquitinated only when the HPV16 E6 was present (Fig. 3E). We subsequently constructed KDM5C K1479R mutant form and performed similar analysis in HCT 116 cells. The K1479R point mutation eliminated most of the polyubiquitination (Fig. 3F). We also cotransfected HCT116 cells with Flag-WT KDM5C or K1479R mutant form and EGFP-HPV16 E6-expressing plasmids, the immunoblot results showed that WT KDM5C but not the K1479R mutant was degraded (Fig. 3G). These results provide strong evidence that KDM5C is a novel target of HPV16 E6 for polyubiquitin-dependent degradation.

HPV16 E6-mediated KDM5C degradation moderately alters the global H3K4 methylation level

We speculated that HPV16 E6-caused KDM5C degradation would result in increased tri- or dimethylation on H3K4. To examine this possibility, we expressed HPV16 E6 in HCT116 cells and examined trimethylation on several histone H3 lysine residues by immunoblots. Among various histone H3 trimethylation marks, H3K4me3, H3K27me3, and H3K36me3, only the H3K4me3 level moderately increased, while H3K27me3 and H3K36me3 remained unchanged in the presence of HPV16 E6 (Fig. 4A). Similar results were also obtained in U2OS cells (Supplementary Fig. S3A). We then compared histone methylations in cervical cancer cell lines. Consistently, the HPV16-positive CaSki cells exhibited a higher H3K4me3 level than the HPV-negative C33A cells (Fig. 4B). Furthermore, we investigated the H3K4 mono- and dimethylation level in response to HPV16 E6 expression in U2OS cells. We found that both H3K4 tri- and dimethylation levels slightly increased, whereas the global H3K4me1 alteration was unchanged (Fig. 4C). However, an effect of HPV16 E6 on the local H3K4me1 distribution could not be ruled out. This issue will be addressed later.

To confirm that KDM5C destabilization alone is responsible for the increased H3K4me3, we ectopically expressed KDM5C in U2OS cells. As shown in Supplementary Fig. S3B, expression of HPV16 E6 increased the H3K4me3 level (lane 2), whereas expression of KDM5C (lane 3), but not the empty vector (lane 4), dramatically decreased H3K4me3. In contrast, ectopic

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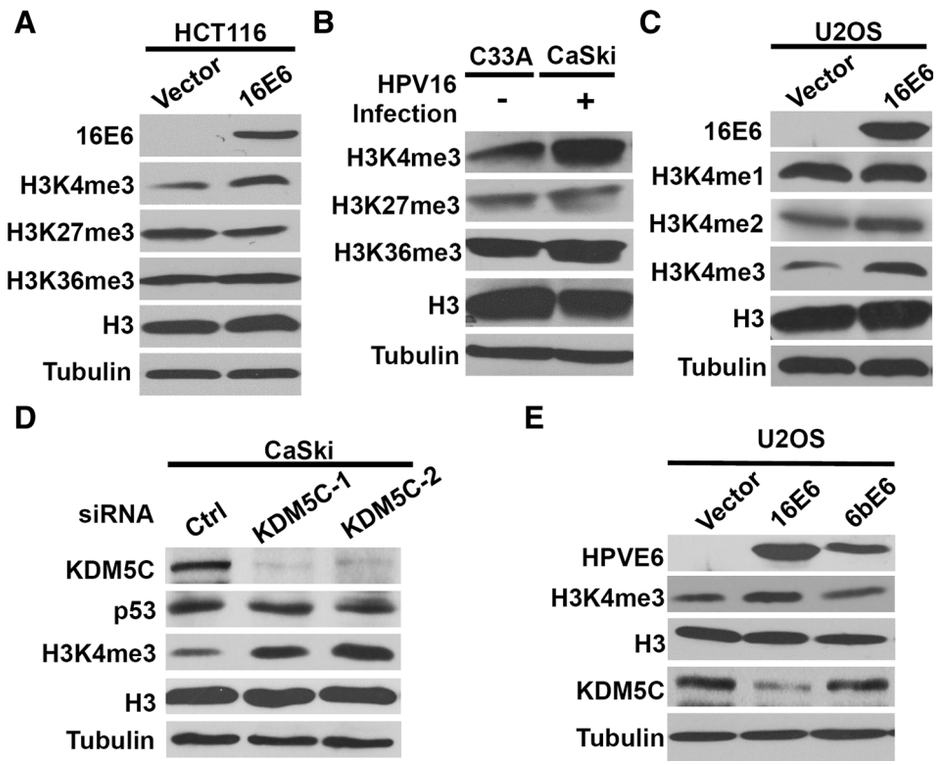

Figure 4.
HR HPV16 E6 moderately alters the global H3K4 methylation level. **A,** HPV16 E6 or vector was ectopically expressed in HCT116 cells. Trimethylation marks of Histone H3 were determined by immunoblot. **B,** Endogenous HPV16 E6 is expressed in CaSki cells, hence the increase in H3K4me3 in comparison with HPV16-negative C33A cells. **C,** H3K4 mono-, dimethylation, and trimethylation levels in response to HPV16 E6 expression in U2OS cells. **D,** Expression change of H3K4me3 upon KDM5C knockdown. siRNA knockdown of KDM5C increases H3K4me3 level, and p53 protein level remains unchanged. **E,** KDM5C and H3K4me3 level analysis in HPV16 E6, the LR HPV6b E6, or vector-overexpressing U2OS cells.

coexpression E6 and p53 did not have additional effect on the level of H3K4me3 (Supplementary Fig. S3C). Thus, the HPV16 E6-mediated KDM5C degradation plays a pivotal role in altered global H3K4 trimethylation. To confirm that KDM5C is an essential regulator of global H3K4me3, we knock down the KDM5C in CaSki cells. The depletion of KDM5C leads to increased H3K4me3 without affecting the p53 protein level (Fig. 4D).

The HR HPV E6 proteins destabilize many host cell proteins (8). In contrast, the "low-risk" (LR) HPV E6 proteins do not appear to possess such ability. To clarify if the HPV16 E6-mediated KDM5C degradation is shared with the LR HPV types, we separately expressed EGFP-HPV16 E6 and EGFP-HPV6b E6 in U2OS cells. Immunoblots with EGFP antibody revealed that 16E6, but not 6bE6, promoted the KDM5C degradation along with an increased in H3K4me3 (Fig. 4E). These results suggest that KDM5C destabilization is probably an important HR HPV E6-mediated oncogenic event.

Restoration of KDM5C significantly suppresses the tumorigenicity of CaSki cervical cancer cells

We next wanted to know the significance of KDM5C destabilization and whether this event contributed to tumorigenesis. To achieve the goal, we used the CaSki-KDM5C stable cell line with elevated KDM5C protein (Fig. 5A). We found that ectopic KDM5C resulted in slightly decreased 16E6/E7 mRNA, but there was no obvious change in 16E6/E7 and p53 protein levels (Supplementary Fig. S4A and S4B). We first examined these cells for several properties *in vitro*. When comparing with the parental cells, CaSki-KDM5C exhibited a reduced growth rate (Fig. 5B), a decreased wound closure (Fig. 5C), and a lowered migration rate in Transwell (Fig. 5D). Lastly, a mouse xenograft tumor model was used to determine the effects of KDM5C overexpression on tumorigenesis *in vivo*. The results showed that compared with CaSki cells containing the vector control, tumors that derived from CaSki-KDM5C grew more slowly and were smaller (Fig. 5E and F). Immunoblots confirmed the elevated KDM5C in CaSki-KDM5C in tumor xenografts without affecting the p53 protein level (Fig. 5G). To confirm the role of KDM5C as a tumor suppressor in cervical cancer, we also generated xenograft by using CaSki cells in which KDM5C was knocked out by using the CRISPR-Cas9. The depletion of KDM5C significantly promotes the tumor growth (Supplementary Fig. S5A–S5D). These results strongly suggest that KDM5C plays an important role in cervical carcinoma growth *in vivo*.

Whole genome ChIP-seq revealed existence of the *EGFR* and *c-MET* super-enhancers in the human cervical cancer cell line

A recent study revealed that KDM5C acts as a key regulator of super-enhancers by controlling dynamics between H3K4me1 and H3K4me3, and that loss of such enhancer surveillance may contribute to tumorigenesis (29). Super-enhancer is a group of densely clustered active enhancers, highly associated with cell identity genes and disease-associated genomic variations (30). As an attribute of being highly active or transcribed, the screening of tumor-specific super-enhancers and the relevant functional study are now of intense interest (23). Our finding *in vitro* and *in vivo* (Fig. 2–5) prompted us to propose that KDM5C restoration may inhibit the tumorigenicity of CaSki cervical cancer cells by dampening specific super-enhancer status.

To test this hypothesis, we screened for the existence of possible super-enhancers by carrying out chromatin immunoprecipitation sequencing (ChIP-seq) analysis of H3K27Ac, one of the most important and characterized features of enhancer, in the CaSki cells, as well as the CaSki-vector control and CaSki-KDM5C cells.

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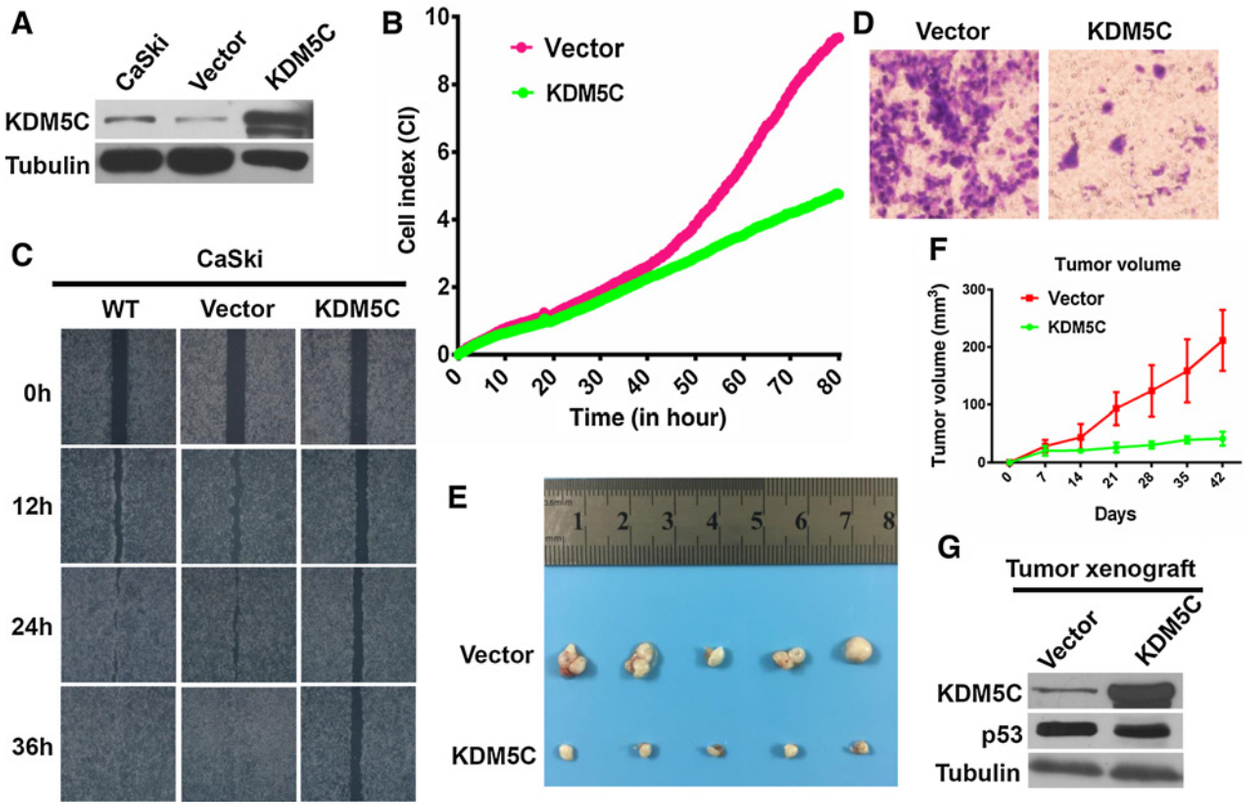

Figure 5.

Restoration of KDM5C inhibits tumorigenesis in cervical carcinoma. **A,** Protein levels of KDM5C in the parental CaSki, CaSki transfected with vector (CaSki-pHAGE), and CaSki-KDM5C cells were examined by immunoblot. **B,** Cell proliferation was determined by using real-time cell proliferation assay. Cervical cancer cell overexpressing KDM5C exhibited a reduced cellular growth rate. **C,** Migrating capability of the parental CaSki, CaSki-vector, and CaSki-KDM5C cells was determined by wound-healing assay. Images were gathered at intervals of 0, 12, 24, 36 hours (magnification, ×40). **D,** Transwell assays of parental CaSki, Caski-vector, and CaSki-KDM5C cells to examine cell invasion. Cells were stained with crystal violet (magnification, ×100). **E** and **F,** Xenograft growth (42 days; $n = 10$) analysis of CaSki-vector and CaSki-KDM5C cells. Representative images of the tumors (**E**) and quantifications of tumor volume (**F**) are shown. The animal protocols were approved by the Animal Welfare Committee of Basic Medical College, Wuhan University. **G,** Immunoblots to examine KDM5C and p53 protein level changes in tumor xenografts.

By using the ROSE algorithm mainly based on the ranking of H3K27Ac intensities (31), we identified a total of 313 super-enhancers in parental cells. Dramatically, we found two proto-oncogenes, *EGFR* and *c-MET*, each contained a super-enhancer. The *EGFR* super-enhancer is located in the first intron while the *c-MET* super-enhancer resides within the *c-MET* gene 5′ region through intron 2 (Fig. 6A; Supplementary Figs. S6 and S7A). In the ChIP-seq results of CaSki-vector and Caski-KDM5C, we also found the same two super-enhancer regions (Fig. 6B), indicating that our determination of *EGFR* and *MET* super-enhancers in these cervical cancer cell lines was reproducible. Our results are highly similar to an early study that three adjacent enhancers clustered within the *EGFR* gene 5′ region down to the intron 1 (32). Moreover, a recent study also identified an *EGFR* super-enhancer in the first intron in A549 lung cancer cells (33).

To validate the results, we compared the human LHCN-M1 cell H3K27Ac ChIP-seq data (from https://www.ncbi.nlm.nih.gov/geo/, GSM2068356) with our data from CaSki cells. The result clearly showed that H3K27Ac signals in these two super-enhancer regions matched well (Supplementary Fig. S7A). In addition, chromatin landscape analysis through dbSUPER (www.bioinfo.au.tsinghua.edu.cn/dbsuper) revealed high levels of H3K27 acetylation (H3K27Ac) across these regions, thus strongly suggesting the existence of super-enhancer (Supplementary Fig. S7B). These results encouraged us to determine the H3K4 methylation status and enhancer RNA (eRNA) expression profiles of the super-enhancers and to explore if KDM5C regulates these super-enhancer activities.

KDM5C regulates cervical cancer cell *EGFR* and c-*MET* expression by modulating their super-enhancer H3K4 methylation dynamics

To determine the whether and how KDM5C may modulate *EGFR* and *c-MET* transcription via their super-enhancers, we performed the ChIP-qPCR of KDM5C. Interestingly, KDM5C were enriched at two distinct selected sites (sites 1 and 2) of the super-enhancer regions of those two genes and the restoration of KDM5C further promoted its enrichment (Fig. 6C and D; Supplementary Fig. S7A), displaying a direct correlation between them. In contrast, the region adjacent to super-enhancer (site 3) showed much lower KDM5C enrichment, and ectopic KDM5C expression failed to stimulate the enrichment. In general, for a

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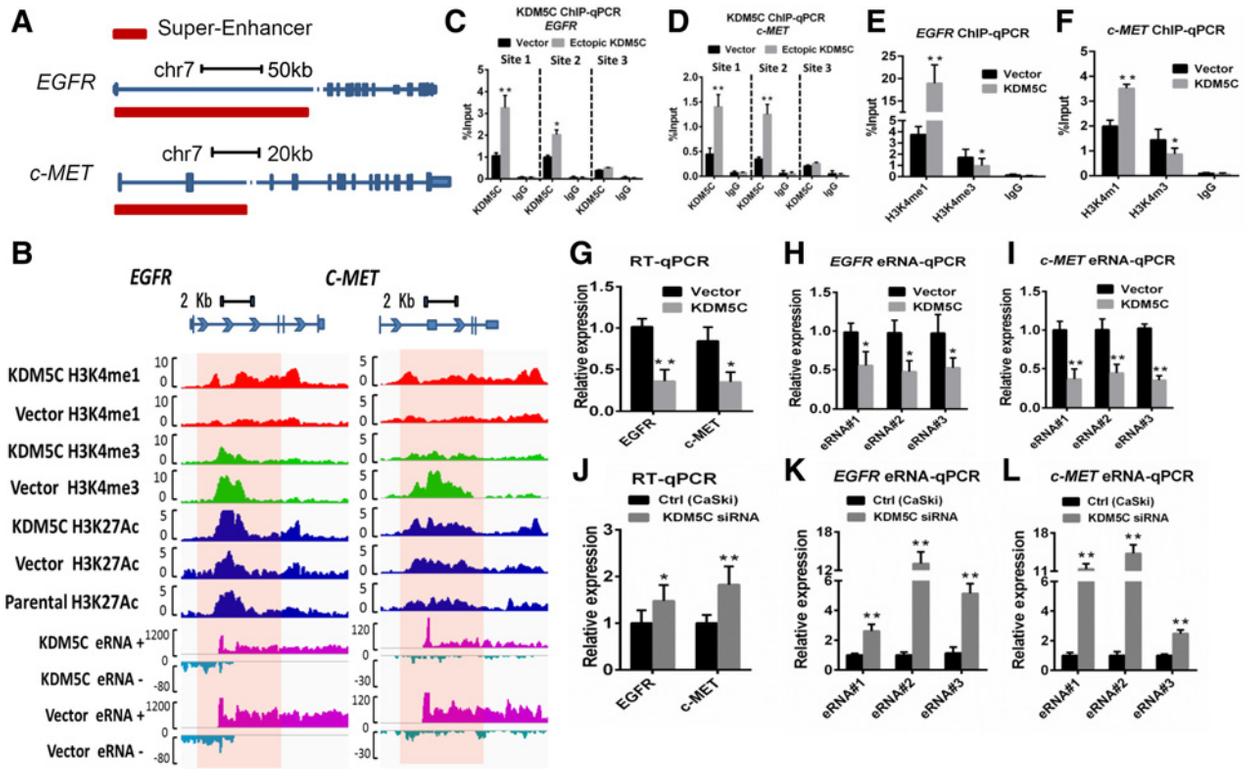

Figure 6.

KDM5C regulates super-enhancers activity and gene expression of the *EGFR* and *c-MET*. **A,** Schematic representation of *EGFR* and *c-MET* super-enhancer. **B,** ChIP-seq result shows H3K4, H3K27 methylation states in parental CaSki, CaSki-vector, and CaSki-KDM5C cells. The RNA-seq results indicate eRNAs decreased at *EGFR* and *c-MET* super-enhancers in CaSki-KDM5C cells. **C** and **D,** KDM5C were enriched at super-enhancer or other regions of *EGFR* and *c-MET* as verified by ChIP-qPCR. **E** and **F,** ChIP-qPCR data show that KDM5C restoration led to H3K4me3/ H3K4me1 alteration of *EGFR* and *c-MET* super-enhancers in CaSki-vector and CaSki-KDM5C cells. **G,** *EGFR* and *c-MET* mRNA levels in CaSki cell with or without KDM5C restoration. **H** and **I,** Ectopic KDM5C expression-mediated eRNA transcription reduction of *EGFR* and *c-MET* genes was confirmed by qPCR of three selected super-enhancer–derived eRNAs in the CaSki-vector and CaSki-KDM5C cells. **J,** mRNA expression of *EGFR* and *c-MET* in CaSki- and KDM5C-depleted CaSki cells was examined by RT-qPCR. **K** and **L,** The eRNA transcription levels at *EGFR* and *c-MET* super-enhancers were examined by qPCR of three selected super-enhancers in parental CaSki- and KDM5C-depleted CaSki cells. In all panels, qPCR data are represented as mean ± SD from three biological replicates. *, $P < 0.05$; **, $P < 0.01$; *t* test.

super-enhancer, H3K4me1 is combined with H3K27Ac to further define active enhancers (34). The dynamics between H3K4me1 versus H3K4me3 is also a determinant in the super-enhancer activity (29). To gain further insight into the mechanism regarding how KDM5C restoration might modulate the proto oncogene expression, we performed ChIP-seq of H3K4me3 and H3K4me1. H3K4me1 overlapped the H3K27Ac signal (Fig. 6B), again validating our super-enhancer analysis. When KDM5C was overexpressed, H3K4me3 decreased while H3K4me1 increased in selected super-enhancer regions (Fig. 6B), in agreement with the known enzymatic activity of KDM5C. The H3K4me3/H3K4me1 alteration was supported by ChIP-qPCR analyses in CaSki derived cells (Fig. 6E and F). The H3K4me3/H3K4me1 dynamics observation implies that KDM5C restoration may have dampened specific super-enhancer status. Our result also revealed that KDM5C restoration led to specific increased H3K4me1 in global super-enhancers rather than in adjacent regions (Supplementary Fig. S8), confirming KDM5C as a specific enhancer regulator.

Other aspects, such as the transcription of nearby gene and bidirectional enhancer RNA (eRNA) transcription, are the important features to determine if the super-enhancer is active or overactive (35). First, in CaSki KDM5C cells, both *EGFR* and *c-MET* mRNA levels were significantly downregulated, indicating that KDM5C indeed negatively regulates their expressions (Fig. 6G). Next, we did RNA-seq to determine the eRNA transcription. Both regions were bidirectionally transcribed into RNA, a well-known feature of active super-enhancers. The data showed that KDM5C restoration greatly decreased eRNAs transcribed from the super-enhancer (Fig. 6B), consistent with the decreased *EGFR* and *c-MET* mRNA expression (Fig. 6G). The reduction in KDM5C-mediated eRNA transcription was also readily confirmed by qPCR of three selected eRNAs (Fig. 6H and I). The H3K4me1/H3K4me3 dynamic change, combined with the results of eRNA analysis, target gene mRNA expression, and KDM5C super-enhancers enrichment, strongly suggests that KDM5C restoration significantly suppresses the *EGFR* and *c-MET* super-enhancer activities.

KDM5C depletion further activates the *EGFR* and *c-MET* super-enhancers

To mimic the HPV16 E6-mediated KDM5C degradation, we knocked down the KDM5C expression by using the siRNA in CaSki cells (Fig. 4D). In contrast to ectopic KDM5C expression,

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the depletion of KDM5C increased the mRNA expression of both *EGFR* and *c-MET* genes (Fig. 6J). In breast cancer cells, the absence of KDM5C promotes several oncogenes expression by switching their super-enhancers status from active to over-active (29). We therefore measured the eRNA transcription from *EGFR* and *c-MET* super-enhancers. As shown in Fig. 6K and L, the three selected super-enhancer–derived eRNAs all exhibited higher transcription levels upon KDM5C depletion, demonstrating the importance of KDM5C in suppressing the expression of the two proto-oncogenes.

Expression of HPV16 E6 activates the super-enhancers by degrading KDM5C

Because HPV16 E6 is able to destabilize KDM5C, a key regulator of super-enhancer, we reasoned that the presence of HPV16 E6 would directly activate the super-enhancers. It follows that HPV16 E6 knockdown in CaSki cells would lead to decreased *EGFR* and *c-MET* gene expression. Because E6 is critical for the survival of cervical cancer cells (36, 37), we can only knock down E6 by a moderate amount. In these experiments, we indeed observed a moderate reduction in *EGFR* and *c-MET* expression (Supplementary Fig. S9A). To substantiate this hypothesis, we stably overexpressed HPV16 E6 in C33A cells. The results show KDM5C degradation and increased expression of *EGFR* and *c-MET* (Fig. 7A and B). We then conducted ChIP-qPCR to determine the KDM5C, H3K4me1, H3K4me3 signals on super-enhancers in these cells. The presence of HPV16 E6 decreased the KDM5C recruitment to super-enhancers of both proto-oncogenes (Fig. 7C), presumably because of the reduced KDM5C protein level. Accordingly, H3K4me1 decreased while H3K4me3 increased (Fig. 7D and E). We suggest that the super-enhancers switched from "active" to "overactive" status, resulting in further elevated *EGFR* and *c-MET* mRNAs. Lastly, we examined eRNA transcription. As indicated in Fig. 7F and G, in the presence of HPV16 E6, the eRNA transcription clearly increased, reminiscent of KDMC5 depletion (Fig. 6). However, when HPV16 E6 and KDM5C were coexpressed in C33A cells, the level of KDM5C protein increased, accompanied by a reduction in *EGFR* and *c-MET* transcription (Fig. 7A and B). Furthermore, the coexpression of KDM5C also reduced the eRNAs expression level (Fig. 7F and G). Consistent with these results, upon knocking down HPV16 E6 in CaSki cells, eRNA transcription was markedly suppressed (Supplementary Fig. S9B and S9C), indicating that HPV16 E6 was able to activate the oncogene super-enhancers by degrading KDM5C.

Because E6 is a multifaceted protein, to rule out the possibility that E6 activate super-enhancers through other proteins, we knocked down KDM5C in HPV-negative cervical cancer cells C33A (Supplementary Fig. S9D), both *EGFR* and *c-MET* gene expression were upregulated upon KDM5C depletion (Supplementary Fig. S9E), as well as their super-enhancers eRNA transcription (Supplementary Fig. S9F and S9G). These results reinforced the idea KDM5C is the key super-enhancer regulator in cervical cancer cells and its degradation by HPV16 E6 activates the super-enhance and elevates the expression of *EGFR* and *c-MET*.

Discussion

The HR HPV E6 and E7 oncoproteins are involved in multiple biochemical activities, including destabilizing tumor suppressors, such as pRB, p53, and TIP60. However, these activities are not able to account for HPV-caused cancers completely. Therefore, it is of interest to identify novel cellular factors that play a role in HPV carcinogenesis. In addition, HPV oncoproteins alter the gene expression, such as *KDM6A* (38), *OGT* (39), *EGFR*, *c-MET*, as well as a number of miRNAs (40), probably via unknown epigenetic mechanisms. For instance, HPV E6 has been shown to target several histone modifying enzymes, such as p300 (41), TIP60, CARM1, PRMT1, and SET7, but until now there was no direct correlation between the host transcription alteration and HPV induced epigenetic change. KDM5C is a novel tumor suppressor identified in renal and breast cancers (29, 42). This protein has been shown to suppress the LCR promoter and inhibit E6/E7 expression by forming a complex with the HPV E2 protein, in a manner highly similar to the E2–TIP60 interaction (27). However, HPV E2 and related proteins are not expressed in HPV cancers in which the viral DNA has integrated into the host chromosomes, disrupting the E2 transcription unit (43, 44).

Here, we show that HPV E6 destabilizes KDM5C in a proteasome-dependent pathway, as has been shown for TIP60 (6). However, unlike TIP60 destabilization by LR and HR HPV E6 proteins, our result reveals that the HR HPV16 E6, but not the LR HPV E6, mediates KDM5C degradation (Fig. 4D). This activity appears to have an important role in tumorigenesis. We demonstrate that the KDM5C restoration via ectopic expression significantly inhibits cervical cancer cells growth in nude mice (Fig. 5). A recent study suggested that loss of KDM5C results in further activation of their target enhancers, thereby promoting tumorigenesis of breast cancer cell (29).

Even though the destabilization of both KDM5C and p53 by E6 is mediated by the E6AP ubiquitin ligase, the domain of E6, critical to these two activities, is different. Using p53 as an internal control in our domain mapping experiment, we showed that full-length HPV16 E6 expression eliminated most of the KDM5C and p53, while the E6AP binding–defective 16E6 mutant Y79N and 16E6 N-terminal domain 1–43 failed to cause the degradation of either KDM5C and p53 (Fig. 2C, lanes 5 and 7). In contrast, the 16E6 mutation F2V/P5R, a previously reported p53 binding–defective mutation, indeed did not destabilize p53; but it was able to destabilize efficiently the KDM5C (lane 3). Similarly, the 16E6 C-terminal domain 44–151 retains the KDM5C interacting activity, resulting in significantly reduced KDM5C; but it was not able to destabilize p53 protein (lane 6). These results suggest that although the HPV16 E6 is capable of mediating both p53 and KDM5C degradation in a E6AP-dependent pattern, distinct HPV 16E6 domains function in these two protein destabilization based on their interaction with p53 or KDM5C, respectively. For p53 degradation, the HPV16 E6 N terminal region is required, while the C-terminal domain is more critical for KDM5C destabilization. Our study also showed that HPV16 E6 does not destabilize KDM5A and KDM5B (Fig. 2A; Supplementary Fig. S2A). KDM5A, B, and C share similar domains in the N-terminus. However, KDM5C C-terminus is shorter and contains only one PHD domain while KDM5A and KDM5B each has two. Interestingly, KDM5C interacts with HPV16 E6 through the C-terminus. Thus, the unique KDM5C C-terminus may help explain why only KDM5C but not its other family members is able to interact with HPV16 E6.

A number of super-enhancers have been identified in various types of cancer; however, they have not been identified in cervical cancer. In elucidating the role and mechanism of KDM5C activity in cervical cancer, we discovered that KDM5C is enriched on *EGFR*

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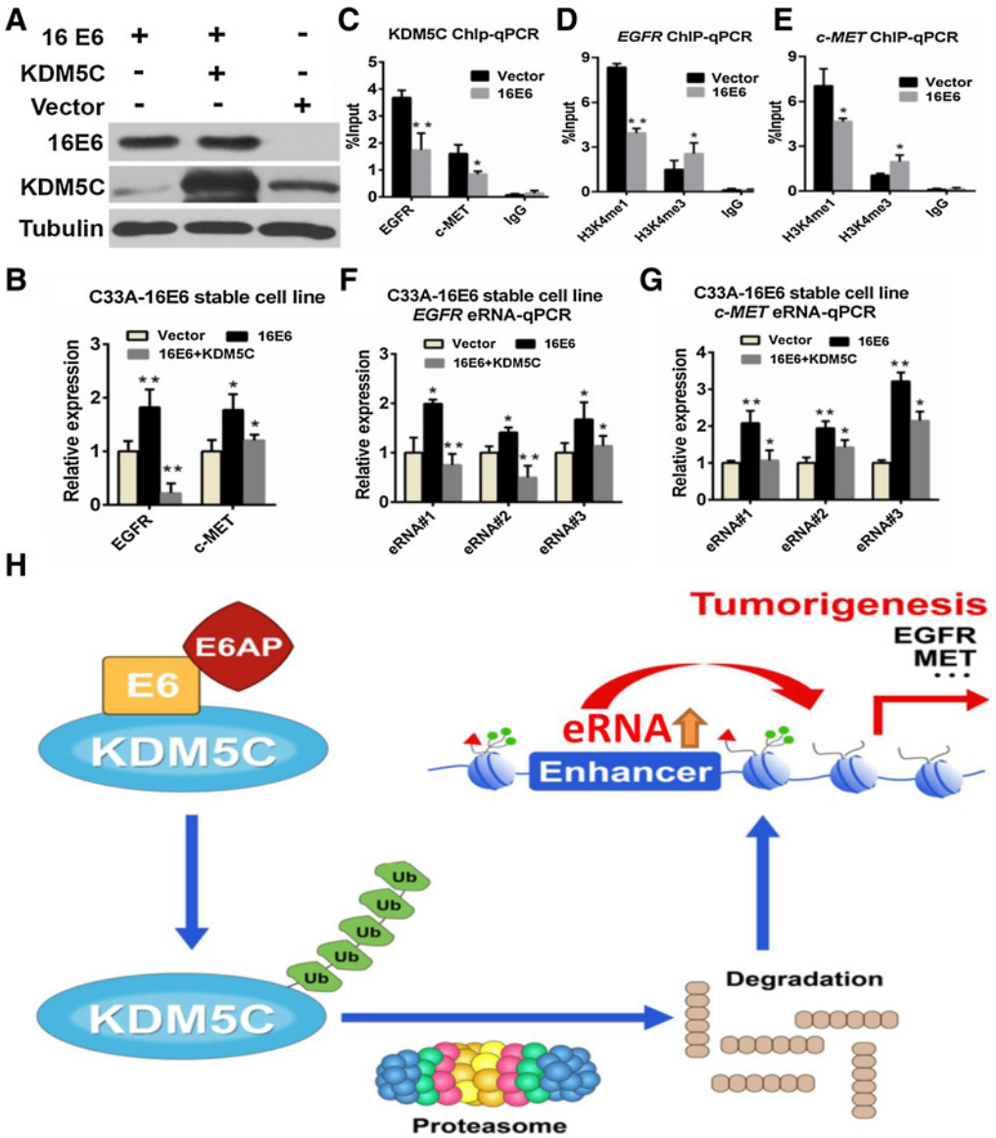

Figure 7.

HPV16 E6 directly activates super-enhancers by degrading KDM5C. **A,** KDM5C protein levels in C33A cells coexpressing HPV16 E6 and KDM5C were examined by immunoblots. **B,** *EGFR* and *c-MET* mRNA levels in stable KDM5C-expressing C33A, C33A-16 E6, C33A-vector were measured by RT-qPCR. **C,** Decreased KDM5C binding at super-enhancer regions of *EGFR* and *c-MET* in C33A-16 E6 cell lines as confirmed by ChIP-qPCR. **D** and **E,** H3K4 methylation state alterations at super-enhancer regions of *EGFR* and *c-MET* in C33A-16 E6 cells relative to C33A-vector cells by ChIP-qPCR. **F** and **G,** eRNA transcription levels at *EGFR* and *c-MET* super-enhancers were examined by qPCR of three selected super-enhancers in the stable C33A-GFP (vector), C33A-16 E6 cells, and KDM5C-expressing C33A-16 E6 cells. In all panels, qPCR data are represented as mean ± SD from three biological replicates. *, $P < 0.05$; **, $P < 0.01$; *t* test. **H,** Model for HPV16 E6 provoked cervical cancer specific super-enhancers activation. HPV16 E6 binds to KDM5C and forms an E6–E6AP–KDM5C complex, which degrades KDM5C in an ubiquitin-dependent manner. As a consequence, key proto-oncogenes increase expression due to the activation of super-enhancers, thereby promoting tumorigenesis.

and *c-MET*, two overexpressed proto-oncogenes in cervical cancer. By systematically analyzing the cervical cancer cells for histone modifications H3K27Ac and H3K4me1/3, as well as the eRNA and mRNA transcription in these two genes, we have established that *EGFR* and *c-MET* each contain a super-enhancer. KDM5C controls the super-enhancers activity by regulating the H3K4me1/H3K4me3 dynamics and eRNA transcription (Fig. 6). Importantly, HPV16 E6 is capable of activating the super-enhancers activity by promoting the KDM5C degradation (Fig. 7). To our knowledge, it is the first report linking the HPV E6 oncoprotein directly to super-enhancer activation via changes in epigenetic modifications. Our study helps explain why the HPV E6 oncoprotein is able to upregulate the host cell oncogene expression.

Taken together, we suggest that overactive super-enhancers may be of one of the driving forces in cervical cancer tumorigenesis, and that the HR HPV16 E6 is responsible for this process.

Collectively, based on our results and previous study, we generate a model as shown in Fig. 7H, HPV16 E6 binds to KDM5C and form an E6–E6AP–KDM5C complex, thereby degrading KDM5C in a polyubiquitin-dependent manner. As a result, the super-enhancers of key proto-oncogenes, *EGFR* and *c-MET*, become highly upregulated, increasing their expressions and promoting tumor cell growth.

Our findings may also provide useful clues to a new mechanism for other pathogen-associated cancers, namely, the regulation of super-enhancer. For example, the hepatitis B virus X protein (HBx) can physically interact with histone acetyltransferase CBP/p300 and promote the expression of PCNA and IL8 (45, 46). Because CPB/p300 catalyze H3K27Ac, a histone hallmark of enhancer, it may worth exploring the effect of the hepatitis B virus X protein on liver cancer super-enhancer formation or activation. In another pathogen-associated cancer, the cytotoxin-associated gene A (CagA) of *H. pylori* has a pathophysiologically important role in gastric carcinogenesis. CagA enhances the expression of DNA methyltransferases 3B (DNMT3B) and histone methyltransferase enhancer of zeste homologue 2 (EZH2), resulting in the attenuation of let-7 expression regulated by histone and DNA methylation (47). Our finding could imply that *H. pylori* proteins possess the potential to influence the generation or modification of gastric cancer super-enhancers.

In summary, we demonstrate that oncogenic HPV16 E6 protein activates cancer super-enhancers. This finding has provided novel insights into virus-induced cancer. We suggest that these discoveries could contribute to the development of targeted cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Chen, J.X. Loo, F. Li
Development of methodology: X. Chen, J.X. Loo, X. Shi, W. Xiong, Y. Guo, M. Yang, F. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Chen, X. Shi, Y. Guo, Y. Jiang, S. Zhong, L. Fu, F. Li
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Chen, J.X. Loo, M. Yang, S. Xia, C. He, F. Li
Writing, review, and/or revision of the manuscript: X. Chen, J.X. Loo, F. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Chen, X. Shi, W. Xiong, M. Zhao, F. Li
Study supervision: X. Chen, F. Li
Other (performed experiments): H. Ke

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