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Activating STING/TBK1 suppresses tumor growth via degrading HPV16/18 E7 oncoproteins in cervical cancer

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Cervical cancer is the most common gynecologic cancer, etiologically related to persistent infection of human papillomavirus (HPV). Both the host innate immunity system and the invading HPV have developed sophisticated and effective mechanisms to counteract each other. As a central innate immune sensing signaling adaptor, stimulator of interferon genes (STING) plays a pivotal role in antiviral and antitumor immunity, while viral oncoproteins E7, especially from HPV16/18, are responsible for cell proliferation in cervical cancer, and can inhibit the activity of STING as reported. In this report, we find that activation of STING-TBK1 (TANK-binding kinase 1) promotes the ubiquitin-proteasome degradation of E7 oncoproteins to suppress cervical cancer growth. Mechanistically, TBK1 is able to phosphorylate HPV16/18 E7 oncoproteins at Ser71/Ser78, promoting the ubiquitination and degradation of E7 oncoproteins by E3 ligase HUWE1. Functionally, activated STING inhibits cervical cancer cell proliferation via down-regulating E7 oncoproteins in a TBK1-dependent manner and potentially synergizes with radiation to achieve better effects for antitumor. Furthermore, either genetically or pharmacologically activation of STING-TBK1 suppresses cervical cancer growth in mice, which is independent on its innate immune defense. In conclusion, our findings represent a new layer of the host innate immune defense against oncovirus and provide that activating STING/TBK1 could be a promising strategy to treat patients with HPV-positive cervical cancer.

Cell Death & Differentiation (2024) 31:78–89; https://doi.org/10.1038/s41418-023-01242-w

INTRODUCTION

Cervical cancer ranks as the fourth most common cancer and the fourth leading cause of cancer death among women worldwide, with approximately 604,000 new cases and 342,000 deaths in 2020 [1]. Persistent infection of high-risk human papillomavirus (HPV), especially HPV16 and HPV18, is the predominant causal factor for development of cervical cancer [2, 3]. Following persistent HPV infection, the double-stranded DNA (dsDNA) virus genomes integrate into the host genome, leading to over-expression of E7, one of the primary oncoproteins [4]. E7 oncoproteins are responsible for cervical carcinogenesis and maintenance through multiple signaling pathways involved in cell proliferation, cell death, and innate immunity [4–8]. For instance, E7 oncoproteins bind and inhibit the tumor suppressor retinoblastoma1 (RB1) via a typical LxCxE domain to release E2F transcription factor for cell cycle progression, and also activates the PI3K/Akt pathway [6, 8]. This pivotal role of E7 oncoproteins in HPV-associated malignity makes them to be attractive therapeutic targets in cervical cancer [8–13]. However, so far there is still no clinically available inhibitor specifically targeting E7 oncoproteins.

Stimulator of interferon genes (STING, also named MPYS/MITA/ERIS), an endoplasmic reticulum (ER) transmembrane protein, serves as an important adaptor for cytosolic DNA sensing pathway during the host antiviral innate immune response [14–16]. In response to HPV infection, cyclic GMP-AMP (cGAMP) synthase (cGAS) recognizes cytosolic dsDNA and produces the second messenger cGAMP to activate STING, which recruits autophosphorylated TANK-binding kinase 1 (TBK1). In turn, TBK1 phosphorylates the C-terminal domains of STING to further recruit interferon regulatory factor 3 (IRF3), which is then translocated into the nucleus to induce expression of type I interferon genes (IFN-I) and the subsequent IFN-stimulated genes, leading to the establishment of host antiviral state [17, 18]. On the other hand, cancer cells often generate micronuclei or cytoplasmic DNA that may trigger cGAS-STING signaling to mount the antitumor immunity response, including driving dendritic cell maturation, antitumor macrophage polarization, T cell priming and activation, natural killer cell activation [19]. The STING pathway is thus proposed as a promising therapeutic target for cancer immunotherapy and may particularly start a new chapter in virus-associated cancer research [20, 21]. Preclinical researches of STING agonists have achieved promising results in a wide range of cancer types, and hence promoted increasing clinical trials, most of which are ongoing [19, 22–25].

Nevertheless, HPVs have evolved to develop means to escape from host innate immunity, and E7 oncoproteins appear to be mostly responsible for the suppression of cGAS-STING pathway [7, 16, 26–28]. For instance, HPV18 E7 oncoprotein could directly

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Received: 17 June 2023 Revised: 8 November 2023 Accepted: 14 November 2023
Published online: 25 November 2023

bind to STING and selectively antagonize STING-triggered innate immune activation in cervical cancer [26, 27]; HPV16 E7 oncoprotein antagonizes the STING pathway via NLRX1-mediated degradation of STING in HPV positive head and neck cancer (HNSCC) [28]. It seems that the interplay between STING and E7 oncoproteins to thwart each other plays an important role in the development of HPV-related cancers. Recently, emerging evidences have demonstrated that STING is involved in regulation of mainstream cellular programs which are not necessarily related to immune response, such as proliferation, differentiation and programmed death [29, 30]. Given the fact that E7 oncoproteins play key roles in cervical cancer proliferation, we wondered whether activated STING may subvert the effect of E7 oncoproteins during their interaction, and eventually alter cervical cancer proliferation.

In this report, we reveal that the TBK1-mediated phosphorylation of HPV16/18 E7 oncoproteins at Ser71/Ser78 promotes their ubiquitination and degradation by E3 ligase HUWE1. Therefore, the STING-TBK1 activation degrades E7 oncoproteins to impede cell proliferation and tumor growth of cervical cancer, which is independent on its innate immune defense, proposing that activating STING/TBK1 may be beneficial for patients with cervical cancer.

MATERIALS AND METHODS

Cell lines and reagents

Human cervical cancer cell lines (Caski, HeLa) and Human embryo kidney (HEK) 293 T cell lines were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC guidelines. *STING*$^{-/-}$ and *TBK1*$^{-/-}$ HeLa cells were gifts kindly provided by Professor Zhengfan Jiang (Peking University). All cell lines were authenticated by the short-tandem repeat profile less than six months before the project was initiated and cultured in DMEM (Gibco) with 10% FBS (ExCell Bio) and 100 U/mL penicillin-streptomycin at 37 °C and 5% CO_2 for no more than one month. STING agonists diABZI, SR-717 and MSA-2 were purchased from MCE. Doxycycline (Dox) was from Sigma and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from MCE. MG132 was from InvivoGen, Bafilomycin A1 was from Selleck and Chloroquine was from MCE.

Plasmid construction

The cDNAs were obtained by PCR amplification and cloned into a pSIN vector with or without a tag (HA, Flag, V5). To generate constructs with specific mutations in HPV18 E7 (S32R, S34A, S78G, S79A, S95A, S103A), HPV16 E7 (S31A, S32A, S63A, S71G, S95A), and TBK1 (S172A, Y325E), forward primers containing mutated sites were used during PCR amplification. STING^{WT}-HA, STING^{V155M}-HA and STING^{R281Q}-HA plasmids were generated as described in our previous work [31], based on which, the corresponding Tet-on plasmids were further generated with a tet-on transactivator. The pcDNA3.1-FH-hHuwe1 constructs [32] were gifts from Professor Genze Shao (Peking University).

The sgRNA oligonucleotides were designed with the web application GUIDES (http://guides.sanjanalab.org/) and inserted into lentiCRISPRv2 plasmids. The siRNAs were synthesized by Guangzhou Ribo Biotechnology Co., Ltd. Sequences of sgRNA and siRNA used in this study were shown in Supplementary Table 1.

All constructs mentioned above were fully verified by Sanger sequencing.

Transfection, lentivirus, and stable cell line construction

In transient transfection experiments, plasmids were introduced into cells utilizing either polyethyleneimine (PEI) (Polysciences) or Lipofectamine 3000. The medium was replenished 6 h after transfection, and subsequent experiments were conducted after 36–48 h. RNAi transfection was performed according to the manufacturer's instructions using RNAiMAX transfection reagent (Thermo Fisher) and 50 nM siRNA.

The production of lentivirus for gene overexpression, sgRNA, and Cas9 expression was conducted as follows. Initially, HEK-293T cells were seeded and allowed to attach for 24 h prior to co-transfection with 3 μg of lentiCRISPRv2-sgRNA/pSin-EF2-cDNA, 2 μg of psPAX2 (gag, pol), 1 μg of pMD2G, and 24 μL of PEI (2 mg/mL). Following 48-h incubation, supernatants were collected and subsequently filtered through 0.45-μm PVDF filters (Millipore). Virus-infected cells were obtained by infecting cells in six-well plates with appropriate viral titers in the presence of 10 μg/mL polybrene (Sigma) and centrifuged at 2000 rpm for 1 h at 37 °C. Finally, stable cell lines were selected by treatment with 0.5 μg/mL puromycin.

Quantitative reverse transcription (qRT) PCR assays

RNA extraction was performed utilizing an RNA extraction kit (TIANGEN) according to the manufacturer's instructions. The resulting total mRNA (1 μg) was subjected to cDNA synthesis utilizing HiScript II Q RT SuperMix for qPCR. Quantitative PCR assays were subsequently performed using SYBR Color qPCR Master Mix (Vazyme) and a LightCycler 480 instrument (Roche). Specific details regarding the qRT-PCR primers utilized in this study are provided in Supplementary Table 2.

Immunoblot and co-immunoprecipitation (Co-IP)

In preparation for Western blot analysis, cells were washed with cold PBS and subsequently lysed on ice in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% NP40), supplemented with Protease Inhibitor Cocktail Set I (Calbiochem; 539131) and Phosphatase Inhibitor Cocktail Set II (Calbiochem; 524625). Lysates were centrifuged at 12,000 rpm for 15 min at 4 °C, and protein quantification was performed using G-250. After mixing with 5x SDS-gel loading buffer and boiling at 100 °C for 5–10 min, equivalent amounts of protein samples were loaded onto SDS-polyacrylamide gels, separated by electrophoresis, and transferred onto PVDF membranes (Millipore) and blocked in PBS containing 5% nonfat milk and 0.1% Tween-20 for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibodies overnight at 4 °C, followed by secondary HRP-conjugated antibody incubation for 1 h at room temperature. Proteins were then detected using High-sig ECL substrate (Tanon) and a MiniChemi Chemiluminescence imager (SAGECREATION, Beijing).

For Co-IP, the supernatants were first incubated with washed anti-V5 agarose (Sigma Chemical Co.) for 2 h (ubiquitination IP) or overnight (phosphorylation IP) at 4 °C, and the precipitates were washed five times with RIPA buffer followed by Western blot analysis. The antibodies used in this study were shown in Supplementary Table 3. Information about the generated phospho-specific antibodies was shown in Supplementary Table 4.

Mass spectrometry (MS) analysis

Affinity purification of Flag-HPV18 E7 oncoprotein was carried out. In brief, HeLa cells were transfected with plasmids encoding Flag-HPV18 E7. The cells were lysed in NETN buffer containing 50 mM β-glycerophosphate, 10 mM NaF, and 1 mg/mL each of pepstatin A and aprotinin. The lysates were centrifuged at 12,000 rpm to remove debris and then incubated with Flag-conjugated beads for 4 h at 4 °C. The beads were washed five times with NETN buffer, the bound proteins were analyzed by SDS-PAGE, and MS was performed by Wininnovate Bio. The immunocomplexes were washed four times with NETN buffer and then subjected to SDS-PAGE and Western blot.

Clone formation assay

HeLa or Caski cells were seeded at a density of 500 or 1000, respectively, per well in 6-well plates and allowed to adhere to the bottom. After 24 h, the cells were treated with Dox (150 ng/mL), diABZI (50 nM, 100 nM or 1 μM), or DMSO (1 μL/mL), or X-rays at the indicated doses, and then cultured for 10–14 days with medium changed every other day. Cell clones were fixed in methanol and stained with 0.1% crystal violet, and those containing more than 50 cells were counted.

MTT assay

Cells (1000/well) were cultured in a 96-well microplate for 24 h and then treated with drugs including Dox (150 ng/mL), diABZI (50 nM, 100 nM or 1 μM), MSA-2 (10 μM), SR7–7 (10 μM), or DMSO (1 μL/mL), or X-rays at the indicated doses. The medium was refreshed every two days to sustain the drug concentration. The cells were incubated with MTT for 4 h and the optical density (OD) was subsequently measured at 490 nm using a microplate reader once per day for continuous 5-6 days.

Tumor xenograft model

Animal studies were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals and the Principles for the Utilization

and Care of Vertebrate Animals and approved by the Animal Research Committee of Sun Yat-sen University Cancer Center (SYSUCC) (Approval no. L102042020100L). NOD-SCID mice (female, 4-6 weeks old, 15–18 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.

Caski cells (3×10^6) inducible expressing Vector, STING^{V155M}, and STING^{R281Q} were subcutaneously inoculated into the right flank of the 6-week-old NOD-SCID mice ($n = 6$ per group). Once the tumor volume reached 50 mm^3, Dox (1 mg/mL) was administered in their drinking water to induce gene expression. Tumor growth was monitored with calipers three times per week, and tumor volume was calculated using the following formula: tumor volume = length × width^2/2 (mm^3). After 27 days, the mice were sacrificed, and the tumors were dissected and evaluated.

A cohort of 6-week-old female NOD-SCID mice ($n = 24$) was randomly assigned to two groups and then inoculated with Caski cells (3×10^6) stably expressing vector or HPV16 E7. Seven days after inoculation, each group of mice was further randomized and divided into two subgroups. The subgroups were treated with 200 µL PBS or 200 µL PBS containing 1.5 mg/kg diABZI by tail intravenous injection three times per week for another 14 days. Tumor volume was recorded biweekly. Twenty-five days post inoculation, the mice were euthanized, and the tumors were excised and weighed.

Statistical analysis

GraphPad Prism (version 8.3.0) software was applied for all statistical analyses. The significance of the differences was assessed by two-tailed Student's *t* test, one-way ANOVA or two-way ANOVA, as appropriate. Data are presented as mean ± SEM. Differences were considered significant when *P* values were <0.05.

RESULTS

Activated STING/TBK1 reduces HPV16/18 E7 oncoproteins in cells

To check whether activated STING may subvert the effect of HPV E7 oncoproteins during their interaction, Caski and HeLa cells, cervical cancer cell lines harboring the integrated HPV16 and HPV18 DNA, respectively, were treated with a potent STING agonist, diABZI [22]. Interestingly, endogenous protein levels of HPV16/18 E7 oncoproteins were markedly decreased in both cell lines upon STING activation as shown in Fig. 1A, B. Likewise, two other STING agonists, SR-717 and MSA-2 [23, 24], could also have the similar results in these two cell lines (Fig. 1C, D). Furthermore, radiotherapy (RT) also decreased endogenous protein levels of HPV16/18 oncoproteins in a dose-dependent manner in Caski and HeLa cells (Fig. 1E, F), as RT is also able to activate STING/TBK1 in cells [20, 33, 34].

Given that STING/TBK1 could be activated by cytoplasmic free DNA during transient transfection, we transiently co-transfected exogenous STING with HPV16 and HPV18 E7 into Caski and HeLa cells, respectively, and the protein levels of exogenous E7 oncoproteins were also obviously decreased (Fig. 1G, H). The decrease of ectopic HPV16/18 E7 oncoproteins by transiently transfected STING was also observed in HEK293T cells (Fig. S1). Moreover, the *Tet-On* regulatory system was employed to generate Caski and HeLa cells stably expressing STING^{WT} (wild-type STING), STING^{V155M} or STING^{R281Q} (two constitutively active STING mutants [35, 36]). Upon doxycycline (Dox) induction, both STING^{V155M} and STING^{R281Q}, but not wild type STING, could decrease endogenous HPV16/18 E7 oncoproteins in these stable cell lines (Fig. 1I, J). Collectively, these results demonstrate that activated STING/TBK1 results in the decrease of HPV16/18 E7 oncoproteins in cells.

Activation of STING/TBK1 promotes the ubiquitination and degradation of HPV16/18 E7 oncoproteins by E3 ligase HUWE1

Next, we sought to investigate how HPV16/18 E7 oncoproteins decrease by STING/TBK1 activation. First, activated STING may down-regulate HPV16/18 E7 oncoproteins at the post-transcriptional level, as mRNA level of HPV18 E7 oncoprotein in cells was marginally changed by either STING^{V155M} or STING^{R281Q}, as well as treatment with diABZI (Fig. S2A, B). Second, the decrease of endogenous HPV16/18 E7 oncoproteins upon diABZI treatment in Caski and HeLa cells was rescued by MG132, a proteasome inhibitor, but not Bafilomycin A1(Baf A1) or Chloroquine (CQ), two lysosome inhibitors (Fig. 2A, B). Consistently, the decrease of exogenous HPV16/18 E7 oncoproteins by transiently co-transfected STING in HEK293T cells was also rescued by MG132, but not Baf A1 or CQ (Fig. S2C, D). Third, the ubiquitylation of exogenous HPV16/18 E7 oncoproteins was increased under transiently co-transfected with STING in Caski, HeLa or HEK293T (Fig. 2C, D, Fig. S2E, F). These results determine that activated STING/TBK1 induces the ubiquitin-proteasome degradation of HPV16/18 E7 oncoproteins in cells.

Then, we tried to identify the E3 ligase responsible for degrading E7 oncoproteins by IP-mass spectrometry (MS) analysis using HeLa cells stably expressing Flag-tagged HPV18 E7. There were three E3 ligases, HUWE1, XIAP and HERC4, among the E7-interacting proteins. However, ectopic HPV18 E7 oncoprotein was only reduced by exogenous HUWE1, but not exogenous XIAP or HERC4, in cells (Fig. 2E, Fig. S3A, B). Likewise, ectopic HPV16 E7 oncoprotein was also reduced by exogenous HUWE1 (Fig. 2F). Consistently, endogenous HPV16/18 E7 oncoproteins levels were elevated in Caski or HeLa cells transfected with sgRNAs targeting HUWE1 (Fig. 2G, H). Moreover, degradation of endogenous HPV18 E7 oncoprotein by either diABZI or RT was abolished by knockdown of HUWE1 in HeLa cells (Fig. 2I, J), and knockdown of HUWE1 decreased the ubiquitination and degradation of HPV18 E7 oncoprotein induced by transient transfection of exogenous STING in both HEK293T and HeLa cells (Fig. 2K, L). Taken together, these results illustrate that E3 ligase HUWE1 is responsible for the ubiquitination and degradation of HPV16/18 E7 oncoproteins upon STING/TBK1 activation.

TBK1-mediated phosphorylation of HPV16/18 E7 oncoproteins promotes their ubiquitination and degradation upon STING activation

Since the TBK1-mediated phosphorylation of STING is the key step for innate immune response in cells [18, 37], we speculated that TBK1 might also be involved in the degradation of HPV16/18 E7 oncoproteins upon STING/TBK1 activation. It was the case, as the decrease of HPV18 E7 oncoprotein by either diABZI or RT was abrogated in HeLa cells deleted either STING (*STING*$^{-/-}$) or TBK1 (*TBK1*$^{-/-}$) (Fig. 3A, B). Therefore, we hypothesized that TBK1 might be the kinase phosphorylating HPV16/18 E7 oncoproteins upon STING activation, as protein stability is commonly regulated by phosphorylation and HPV16/18 E7 oncoproteins had been reported to be phosphorylated [6, 38]. Indeed, exogenous TBK1 could decrease exogenous HPV16/18 E7 oncoproteins in either Caski or HeLa cells (Fig. 3C, D), which was dependent on the TBK1 kinase activity, as TBK1^{S172A} and TBK1^{Y325E}, two kinase-dead (KD) mutants of TBK1 [39], were unable to decrease ectopic HPV16/18 E7 oncoproteins in HEK293T cells (Fig. S4A, B).

We next sought to investigate whether TBK1 can phosphorylate E7 oncoproteins. As shown in Fig. S4C–F, anti-p-serine antibody, but not anti-p-threonine, was detected in cells co-transfected TBK1, but not TBK1^{S172A}, with either HPV16 or HPV18 E7, indicating that TBK1 is able to phosphorylate one or some serine residues of HPV16/18 E7 oncoproteins. Furthermore, TBK1, but not TBK1^{S172A}, could enhance the ubiquitylation of either HPV16 or HPV18 E7 oncoprotein in cells (Fig. 3E, F).

Then, five serine residue mutants of HPV16 E7 protein (S31A, S32A, S63A, S71G and S95A) and six serine residue mutants of HPV18 E7 protein (S32R, S34A, S78G, S79A, S95A and S103A) were generated. As shown in Fig. S5A, B, neither the S71G mutant of HPV16 E7 oncoprotein nor the S78G mutant of HPV18 E7 oncoprotein was down-regulated by exogenous TBK1 in cells.

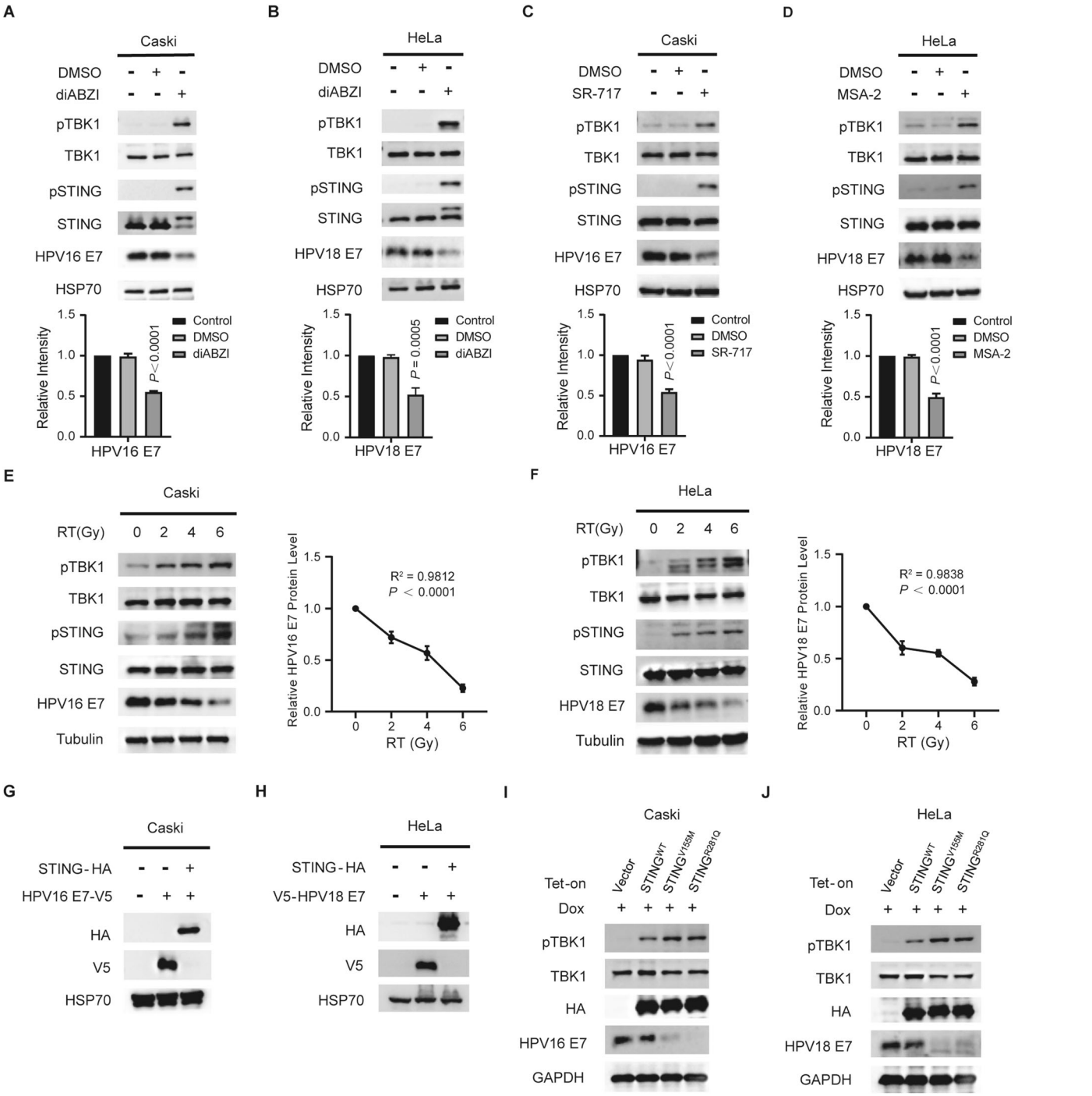

Fig. 1 Activation of STING reduces HPV16/18 E7 oncoproteins in cells. Caski/HeLa cells were treated with DMSO, 100 nM diABZI (**A**, **B**), 10 μM SR-717 (**C**) or 10 μM MSA-2 (**D**) as indicated for 6 h, and then were subjected to Western blot. The protein levels of HPV16/18 E7 oncoproteins were then quantified (mean ± SEM. $n = 3$. two-tailed Student t test. ns: no significance). **E**, **F** Caski or HeLa cells were treated with different doses of X-ray irradiation, and then were subjected to Western blot after 3 days. The protein levels of HPV16/18 E7 oncoproteins corresponding to the radiation dose gradient were monitored by line chart (mean ± SEM. $n = 3$. one-way ANOVA). **G**, **H** Caski and HeLa cells were transiently co-transfected with the indicated plasmids for 48 h, and then were subjected to Western blot. **I**, **J** Caski or HeLa cells inducible expressing $STING^{WT}$, $STING^{V155M}$ or $STING^{R281Q}$ as indicated were treated with 150 ng/mL doxycycline (Dox) for 24 h, and then were subjected to Western blot. These results are repeated of three independent experiments.

Moreover, TBK1-mediated serine phosphorylation of either the S71G mutant of HPV16 E7 oncoprotein or the S78G mutant of HPV18 E7 oncoprotein could not be detected in cells (Fig. S5C, D). These results reveal that serine 71 of HPV16 E7 oncoprotein and serine 78 of HPV18 E7 oncoprotein were the dominant phosphorylation sites by TBK1 in cells.

Using the special phosphorylation antibodies we generated, anti-p-16E7-S71 and anti-p-18E7-S78, as shown in Fig. 3G, H, phosphorylation at serine 71 of HPV16 E7 oncoprotein was detected in cells co-transfected TBK1 with HPV16 E7, but not its S71G mutant, and phosphorylation at serine 78 of HPV18 E7 oncoprotein was also detected in cells co-

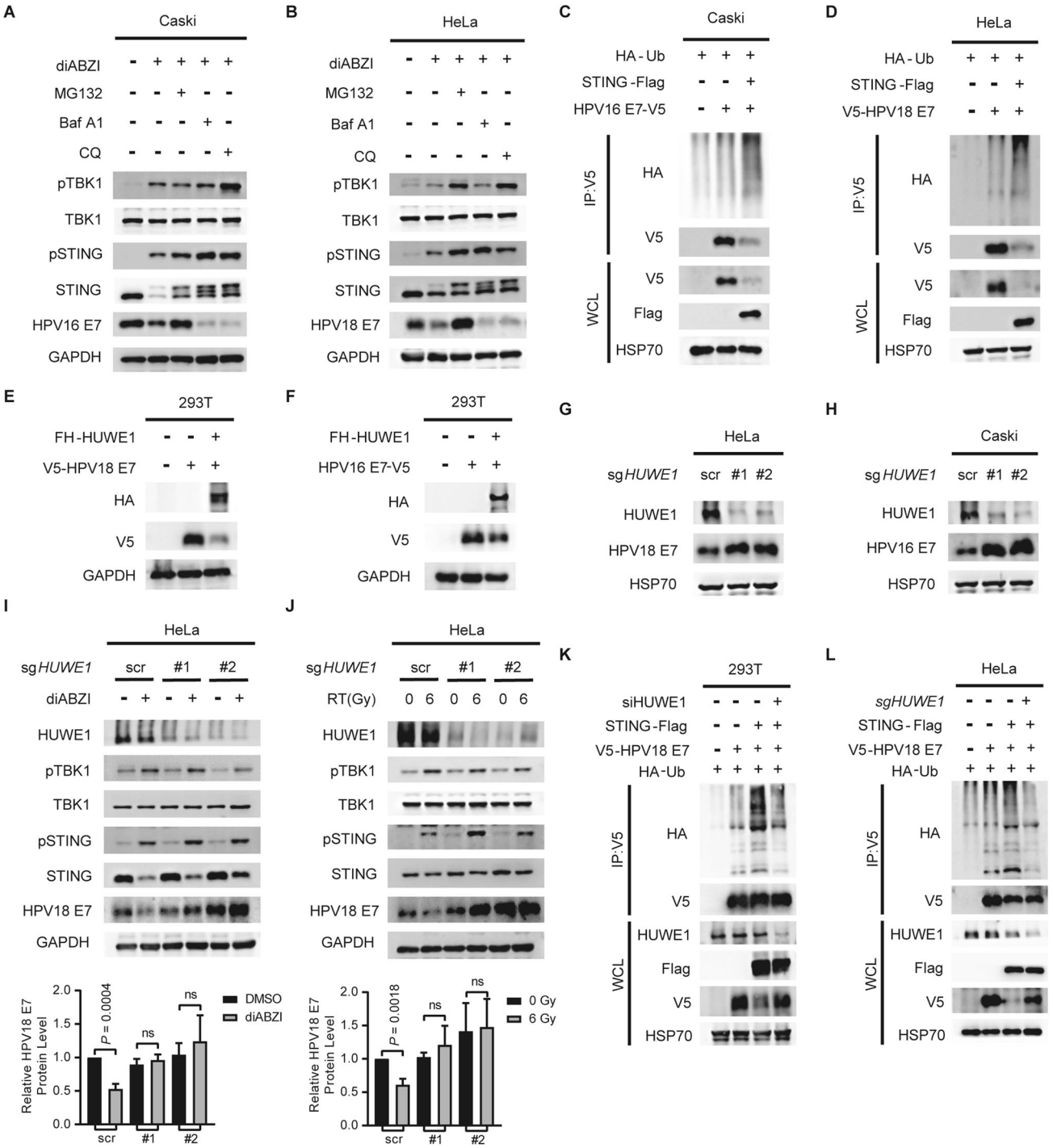

Fig. 2 Activation of STING promotes the ubiquitination and degradation of HPV16/18 E7 oncoproteins by E3 ligase HUWE1. A, **B** Caski or HeLa cells were treated with or without diABZI (100 nM), MG132 (10 μM), Bafilomycin A1 (Baf A1, 200 nM) and Chloroquine (CQ, 40 μM) for 6 h as indicated, and then were subjected to Western blot. **C**, **D** Caski and HeLa cells transiently co-transfected with the indicated plasmids for 48 h were incubated with 5 μM MG132 for 6 h, and then subjected to immunoprecipitation (IP) using anti-V5 antibody followed by Western blot. **E**, **F** HEK293T cells transiently co-transfected with the indicated plasmids for 48 h were analyzed by Western blot. **G**, **H** *HUWE1*-KO HeLa or Caski cells were analyzed by Western blot. *HUWE1*-KO HeLa cells were treated with or without diABZI (1 μM) for 6 h (**I**) followed by Western blot analysis, or were treated with or without 6-Gy X-ray irradiation (**J**), and then were subjected to Western blot after 3 days. Quantification results of the corresponding HPV18 E7 protein level were shown (mean ± SEM. $n = 3$. two-tailed Student *t* test. ns: no significance). HEK293T cells (**K**), HeLa or *HUWE1*-KO HeLa cells (**L**) transiently co-transfected with the indicated plasmids for 48 h were incubated with 5 μM MG132 for 6 h, and then subjected to IP using anti-V5 antibody followed by Western blot. These results are repeated of three independent experiments.

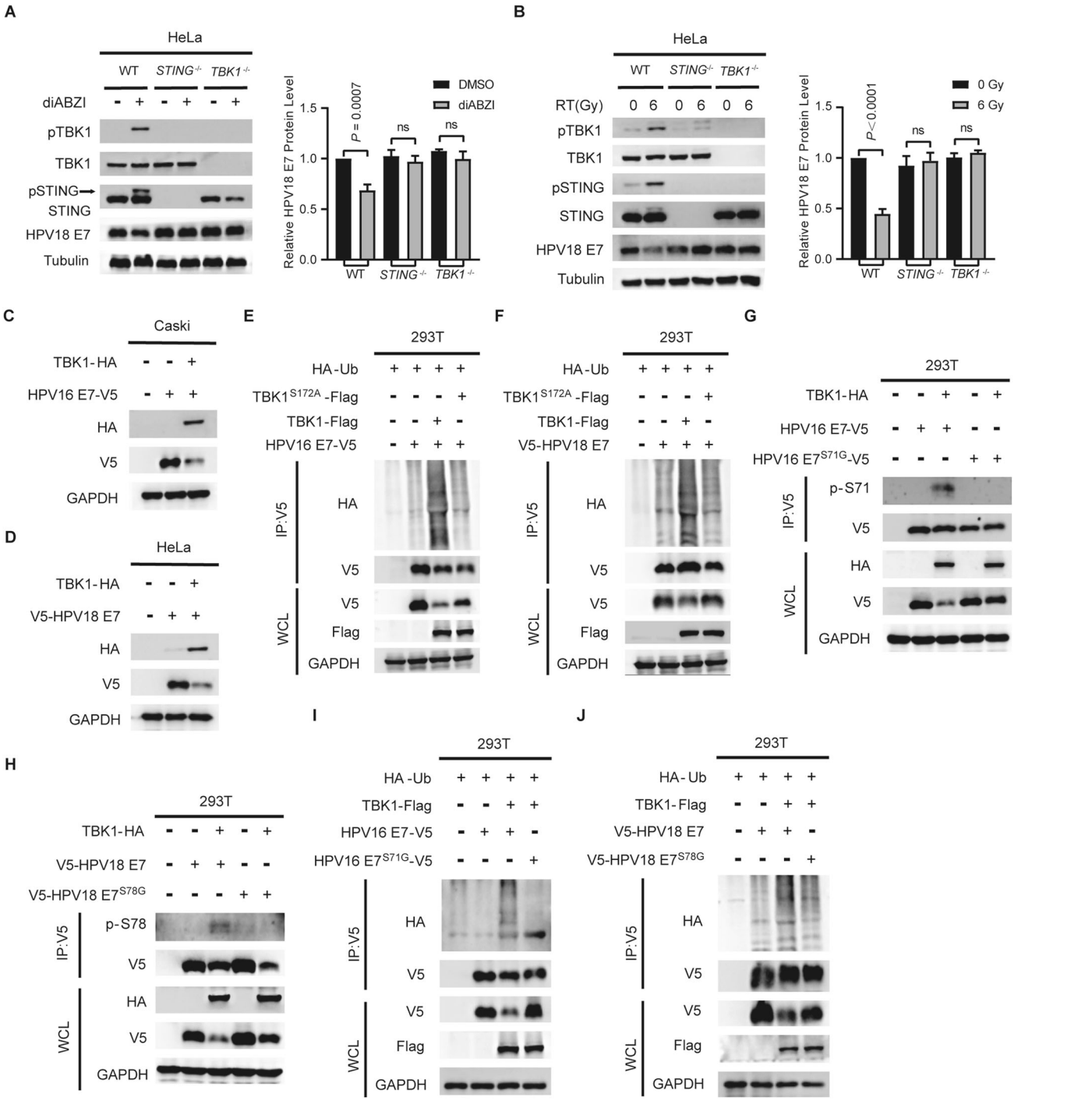

Fig. 3 TBK1-mediated phosphorylation of HPV16/18 E7 oncoproteins promotes their ubiquitination and degradation upon STING activation. WT, *STING*$^{-/-}$ and *TBK1*$^{-/-}$ HeLa cells were treated with or without diABZI (1 μM) for 6 h (**A**) followed by Western blot analysis, or were treated with or without 6-Gy X-ray irradiation (**B**), and then were subjected to Western blot after 3 days. Quantification results of the corresponding HPV18 E7 protein level were shown (mean ± SEM. $n = 3$. two-tailed Student *t* test. ns: no significance). **C**, **D** Caski or HeLa cells transiently co-transfected with the indicated plasmids for 48 h were analyzed by Western blot. **E**, **F** HEK293T cells transiently co-transfected with the indicated plasmids for 36 h were incubated with 5 μM MG132 for 6 h, and then subjected to IP using anti-V5 antibody followed by Western blot. HEK293T cells were transiently cotransfected TBK1 with the indicated plasmids of HPV16 (**G**) or HPV18 E7 (**H**) for 36 h. Cell lysates were subjected to IP with anti-V5-agarose and then analyzed by Western blot. **I**, **J** HEK293T cells transiently co-transfected with the indicated plasmids for 36 h were incubated with 5 μM MG132 for 6 h, and then subjected to IP using anti-V5 antibody followed by Western blot. These results are repeated of three independent experiments.

transfected TBK1 with HPV18 E7, but not its S78G mutant. Consistently, the TBK1-mediated ubiquitination at either the S71G mutant of HPV16 E7 oncoprotein or the S78G mutant of HPV18 E7 oncoprotein was abolished in cells (Fig. 3I, J). Collectively, these results reveal that Ser71 and Ser78 phosphorylation of HPV16 and HPV18 E7 oncoproteins by TBK1 promotes their ubiquitin-proteasome degradation, respectively.

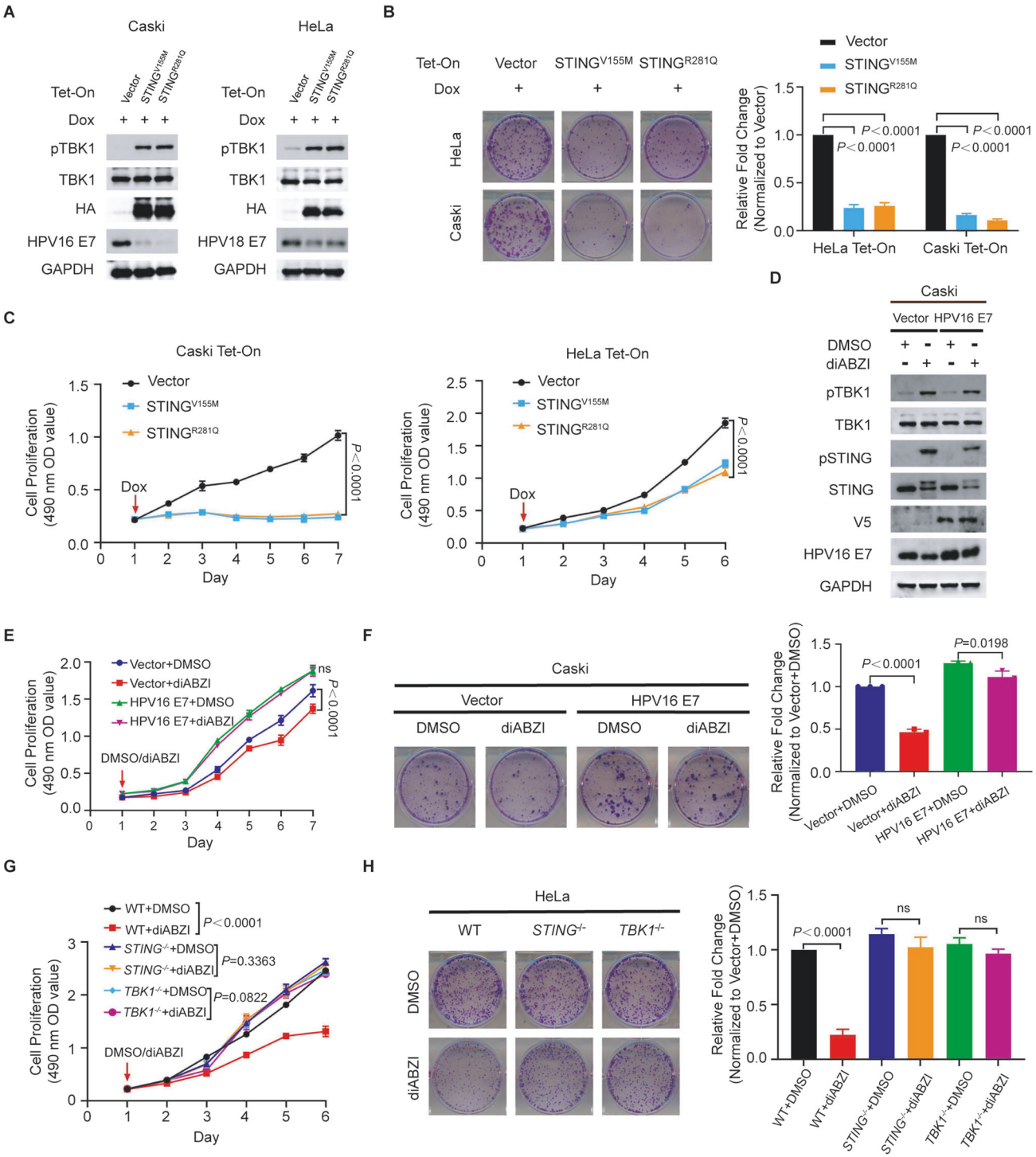

Fig. 4 Activated STING inhibits cell proliferation of cervical cancer cells by degrading E7 oncoproteins in a TBK1-dependent manner. **A** Caski or HeLa cells inducible expressing vector, STING^{V155M} or STING^{R281Q} were treated with 150 ng/mL Dox for 24 h, and were then subjected to Western blot. **B** The indicated cells in (**A**) were treated with 150 ng/mL Dox with medium refreshed every other day. The quantitative analyses of colony-formation assay were shown on the right (mean ± SEM. $n = 3$. two-tailed Student t test), and the representative images were shown on the left. **C** The indicated cells in (**A**) were treated with 150 ng/mL Dox with medium refreshed every other day, and then measured by MTT assay at different time points (mean ± SEM. $n = 6$. two-way ANOVA). **D** Caski cells stably expressing vector or exogenous HPV16 E7 were treated with DMSO or 100 nM diABZI for 6 h, and then subjected to Western blot. **E** The indicated cells in (**D**) were treated with DMSO or diABZI, and then were measured by MTT assay at different time points (mean ± SEM. $n = 6$. two-way ANOVA). **F** The indicated cells in (**D**) were treated with DMSO or diABZI for 14 days with medium changed every 2–3 days. The quantitative analyses of colony-formation assay were shown on the right (mean ± SEM. $n = 3$. two-tailed Student t test), and the representative images were shown on the left. **G** WT, *STING*$^{-/-}$ and *TBK1*$^{-/-}$HeLa cells, as indicated in Fig. 3A, were treated with DMSO or 1 μM diABZI, and then measured by MTT assay at the indicated time points (mean ± SEM. $n = 6$. two-way ANOVA). **H** The indicated cells in (**G**) were treated with DMSO or diABZI for 14 days with medium changed every 2–3 days. The quantitative analyses of colony-formation assay were shown on the right (mean ± SEM. $n = 3$. two-tailed Student t test), and the representative images were shown on the left.

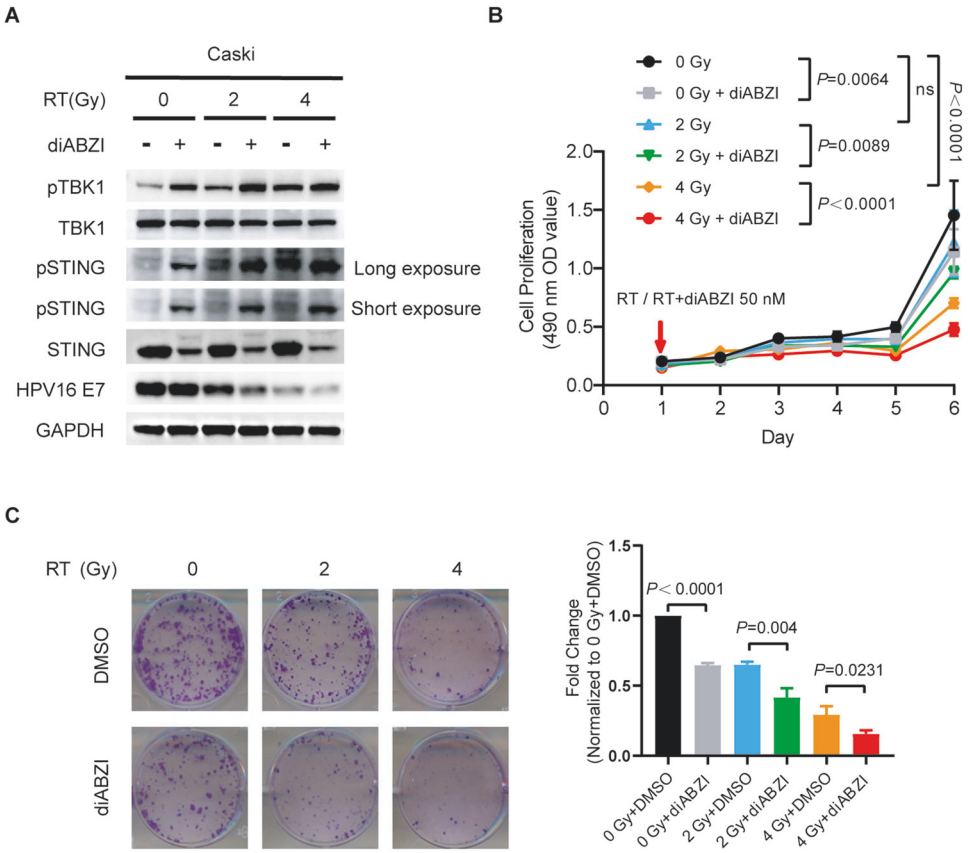

Fig. 5 STING agonist enhances the inhibitory effects of radiation on HPV16 E7 oncoprotein, cell proliferation and clone formation in Caski cells. A Caski cells were treated with or without 50 nM diABZI plus different doses of X-ray irradiation as indicated and then analyzed by Western blot after 3 days. **B** Caski cells were treated with or without 50 nM diABZI plus different doses of X-ray irradiation and then analyzed by MTT assay at the indicated times (mean ± SEM. $n = 6$. two-way ANOVA. ns: no significance). **C** Caski cells were treated with or without 50 nM diABZI for 14 days plus different doses of X-ray irradiation given at the first day. The quantitative analyses of colony-formation assay were shown on the right (mean ± SEM. $n = 3$. two-tailed Student t test), and the representative images were shown on the left.

Activated STING/TBK1 inhibits cell proliferation of cervical cancer cells by degrading E7 oncoproteins in a TBK1-dependent manner

Given that the predominant HPV E7 oncoproteins promote proliferation and growth of cervical cancer, we speculated that activation of STING might impair cell proliferation and tumor growth in cervical cancer by degrading E7 oncoproteins. As shown in Fig. 4A–C, the Dox-induced $STING^{V155M}$ or $STING^{R281Q}$ dramatically decreased the E7 proteins and suppressed cell proliferation and clone formation in Caski and HeLa cells. Three different STING agonists, diABZI, SR717 and MSA-2, could also reduce cell proliferation in Caski and HeLa cells (Fig. S6A, B). Moreover, the diABZI-induced inhibition on both cell proliferation and clone formation was rescued in Caski cells stably overexpressing HPV16 E7 oncoprotein (Fig. 4D–F), and diABZI had no effect on either cell proliferation or clone formation in both $STING^{-/-}$ and $TBK1^{-/-}$ HeLa cells (Fig. 4G, H). These results illustrate that activated STING impedes cell proliferation of cervical cancer cells via the TBK1-induced degradation of E7 oncoproteins.

Since 80% of cervical cancer patients are treated with RT in clinic during their disease courses and RT could decrease E7 oncoproteins by activating STING/TBK1 (Fig. 1E, F), we sought to investigate whether STING agonist can enhance the effect of RT on cervical cancer cell proliferation. As shown in Fig. 5A–C, the combination of diABZI with RT was more effective in decreasing E7 protein level and suppressing cell proliferation and clone formation compared to either diABZI or RT alone. These results indicate that STING agonist plus a low-dose RT may achieve the similar inhibition of a high-dose RT on growth of cervical cancer. For example, such effects of the 2 Gy X-ray plus diABZI were similar to those of the 4 Gy X-ray alone, suggesting that such a combination would enhance the therapeutic effect of RT on cervical cancer patients or substitute for high-dose RT which may cause severe damage to normal tissues or organs.

STING/TBK1 activation inhibits tumor growth of HPV-infected cervical cancer via degrading E7 oncoproteins

In recent years, pharmacologic activation of STING, as a potential immunotherapy for cancer treatment, has been extensively studied in preclinical models and clinical trials. We were very curious to determine whether activation of STING can specifically inhibit the growth of HPV-infected cervical cancer through directly degrading E7 oncoproteins besides its enhancing antitumor immunity. In order to avoid the impact of STING-mediated antitumor immunity, the NOD-SCID mouse model was used to assess the effect of activated STING/TBK1 on tumor growth in vivo using Caski cells stably expressing vector, $STING^{V155M}$ or $STING^{R281Q}$ with the *Tet-On* regulatory system (Fig. 4A). Upon Dox induction, both $STING^{V155M}$ and $STING^{R281Q}$ could strongly reduce the tumor sizes, volumes and weights compared with the vector group (Fig. 6A–D).

As shown in Fig. 6E–H, the tumor volumes and weights were slightly increased in NOD-SCID mice bearing Caski cells stably overexpressing HPV16 E7 compared with those stably expressing vector, and the tumor volumes and weights were impaired by diABZI in NOD-SCID mice bearing Caski cells stably expressing

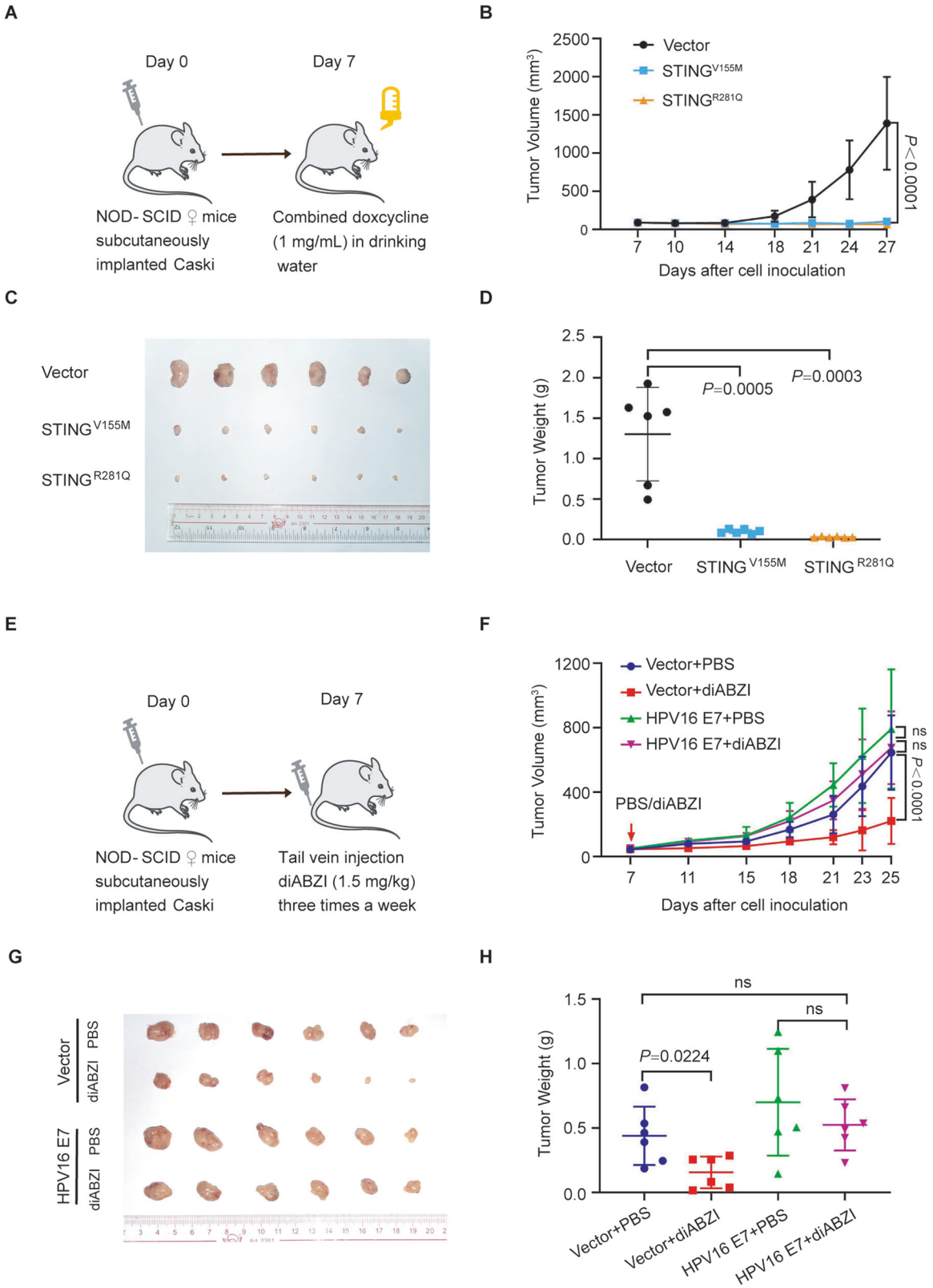

Fig. 6 STING activation inhibits tumor growth of HPV-infected cervical cancer via degrading E7 oncoproteins. A–D Caski cells inducible expressing vector, STING^{V155M} or STING^{R281Q} were inoculated subcutaneously into NOD-SCID mice for a week followed by induction of Dox (1 mg/mL combined in drinking water). The xenografts were excised (**C**) and tumor volume was monitored 3 times a week (mean ± SEM. $n = 6$. two-way ANOVA) (**B**). Tumor weight on Day 27 after transplantation was shown (mean ± SEM. $n = 6$. two-tailed Student t test) (**D**). **E–H** NOD-SCID mice were implanted subcutaneously with Caski stably expressing vector or exogenous HPV16 E7 for a week followed by tail vein injection of PBS or 1.5 mg/kg diABZI 3 times a week. Dissected tumors for xenograft experiments were shown (**G**). Visible tumors were measured every 2–4 days to generate the tumor growth curve (mean ± SEM. $n = 6$. two-way ANOVA) (**F**). Tumor weights on Day 25 after transplantation were calculated (mean ± SEM. $n = 6$. two-tailed Student t test) (**H**).

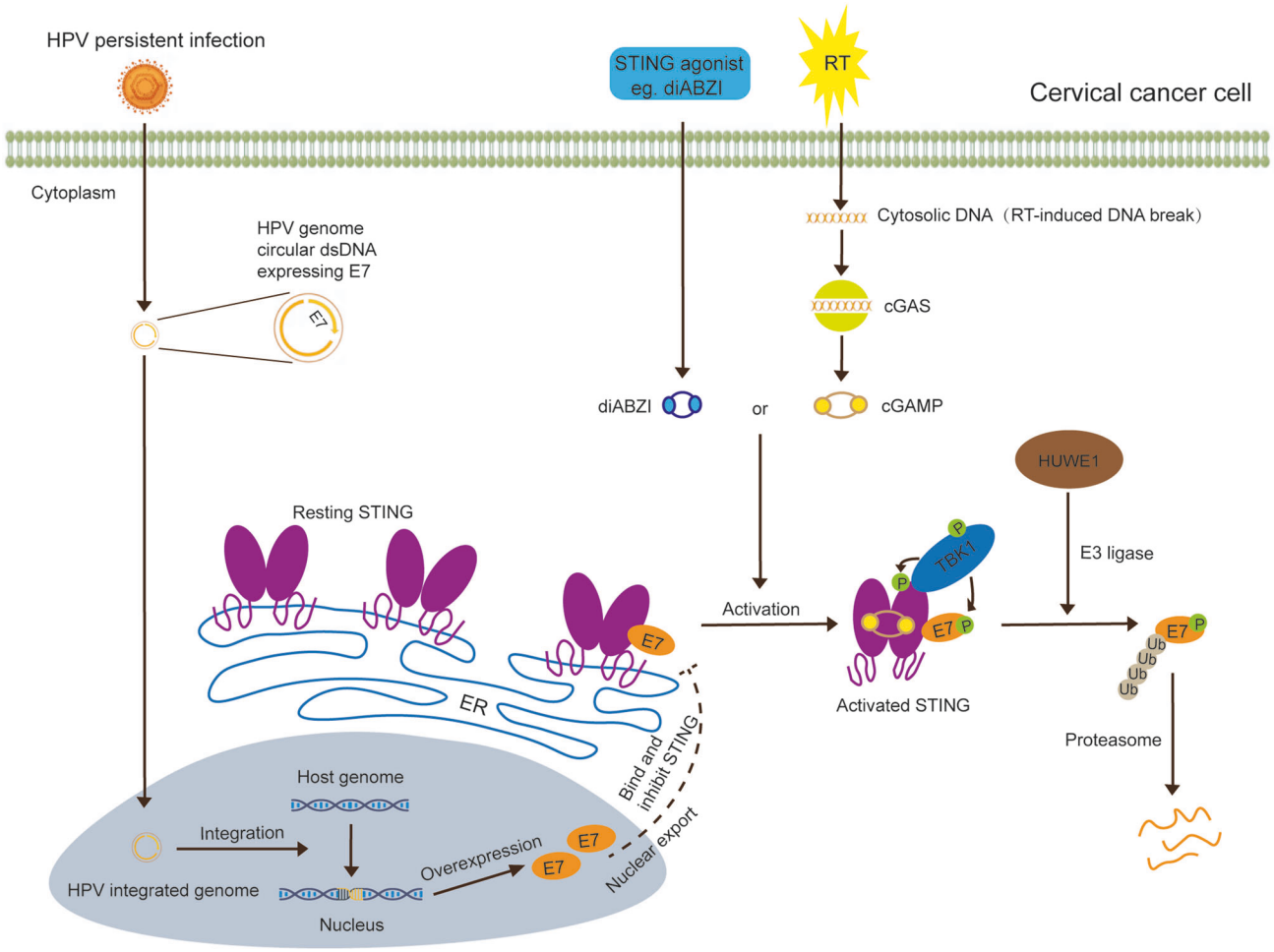

Fig. 7 The proposed model for the crosstalk between STING/TBK1 and HPV E7 oncoproteins in HPV-positive cervical cancer. Cervical carcinogenesis is closely related to the persistent infection of HPV, during which the HPV genome, circular double-stranded DNA (dsDNA), would integrate into the host genome, leading to overexpression of HPV E7 oncoproteins. E7 oncoproteins are exported out of the nucleus dependent on its nuclear export sequences, bind and inhibit the resting STING in endoplasmic reticulum (ER) to attenuate its activation. While once upon activation, such as administration of STING agonist diABZI or radiotherapy (RT), STING dimerizes and translocates to recruit TBK1, which in turn phosphorylates STING and the E7 oncoproteins bound to STING, consequently, the E7 oncoproteins are ubiquitinated and degraded by E3 ligase HUWE1, thereby inhibiting proliferation of cervical cancer cells.

vector, but not in those bearing Caski cells stably overexpressing HPV16 E7. These in vivo results were consistent with that overexpression of HPV16 E7 rescued the diABZI-induced inhibition of cell proliferation in vitro (Fig. 4D–F). Collectively, these results demonstrate that STING activation can inhibit tumor growth of HPV-infected cervical cancer via degrading E7 oncoproteins.

DISCUSSION

The endless war between the host innate immune system and the invading HPV has equipped both with sophisticated and effective mechanisms to thwart each other, during which the crosstalk between STING and HPV E7 oncoproteins has recently emerged to play a critical role in cervical cancer progression [7]. HPV18 E7 oncoprotein has been reported to bind and inhibit STING to escape innate immune surveillance [26, 27]. Intriguingly, in this report we show for the first time that activation of STING/TBK1 promotes the degradation of HPV16/18 E7 oncoproteins to inhibit tumor growth of cervical cancer, which is independent on the innate immunity of STING/TBK1. A schematic illustration of the main findings is shown in Fig. 7. Conceptually, our findings provide a new layer of the host innate immune defense against oncovirus, and propose that activating STING/TBK1 could be a promising therapeutic strategy to treat patients with cervical cancer.

HPV16/18 E7 oncoproteins are mainly resided in nuclear but may present in cytoplasm with a relatively low steady-state level [6], and HPV18 E7 oncoprotein has been reported to bind STING resulting in the inhibition on STING activation to subvert the host innate immunity [26]. On the other hand, here we found that upon activation, STING dimerizes and recruits TBK1 to phosphorylate HPV16/18 E7 oncoproteins at Ser71/Ser78, which in turn promotes the ubiquitination and degradation of E7 oncoproteins by HUWE1, as illustrated in Fig. 7. This is consistent with the literature, showing that HPV16 E7 oncoprotein could be phosphorylated at Ser71 by an unknown protein kinase [38], and that the Ser71 of HPV16 E7 and Ser78 of HPV18 are relatively conserved among various HPV types [11].

Apart from the well-known antiviral and antitumor immunity, STING can suppress cell proliferation via regulating the cell cycle or enhancing the anti-tumor immune response [29, 40]. In this report, we found that STING activation inhibits cell proliferation and tumor growth of cervical cancer via down-regulating E7 oncoproteins, indicating another strategy that STING utilizes to inhibit HPV-related cervical carcinogenesis and progression. Furthermore, our results revealed that the combination of diABZI

with RT was more effective in suppressing cell proliferation compared to either diABZI or RT alone. Based on that targeting HPV16/18 E7 oncoproteins has been verified to be effective in treating cervical cancer [8–13] and that multiple STING agonists are going on clinical trials, we highly recommend that the clinical trials of combination of STING agonists with RT should be set up for treating patients with cervical cancer.

In addition, various therapeutic vaccines, mainly targeting HPV E7 oncoproteins have been developed and tested in preclinical and clinical trials [41], and STING agonists have been recently found to possess a broad spectrum of vaccine adjuvant activities in antiviral defense and antitumor immunity, especially during the global pandemic of coronavirus disease of 2019 (COVID-19) [42, 43]. We propose that STING agonists may be utilized as the adjuvant for HPV therapeutic vaccines to treat HPV-mediated cervical cancer, as they could have direct effects on tumor cells by degrading HPV E7 oncoproteins, as well as indirect effects via immune response activation.

In summary, this study delineates a novel antiviral and antitumor strategy by which a central innate immune sensing signaling adaptor STING functions as a powerful "degrader" of HPV E7 oncoproteins. We identify the ubiquitin-proteasome degradation of E7 following activation of STING-TBK1 signaling as a potential intervention point to suppress the growth of cervical cancer. Our study may enlighten new strategies to treat existing cervical cancer and potential therapeutic vaccination approach to prevent the HPV-related carcinogenesis.

DATA AVAILABILITY

The data generated in this study are available within the article and its supplementary data files. The online version contains supplementary material. Any additional information related to this work is available from the corresponding author on reasonable request. The authenticity of this manuscript was validated by uploading the key raw data to the Research Data Deposit public platform (www.researchdata.org.cn).

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ACKNOWLEDGEMENTS

We thank Dr. Ying Sun (Sun Yat-sen University Cancer Center) for helpful suggestions. We thank members of Dr. Kang's laboratory for providing helpful suggestions on this study. This work was supported by the National Key R&D Program of China (2021YFA1300601), and the grants from the National Natural Science Foundation of China (82002428, 81972430, 82103155, 32100544, 82341015, 82030090)

AUTHOR CONTRIBUTIONS

XH, TK and YG conceived the project, designed the experiments and wrote the manuscript. XH, YG, LH and BX performed most of the experiments and analyzed the data. XH, YO, FC and JL performed the statistical analyses. XH, YG, LH and XZ performed the animal experiments. DW, YW and RZ assisted with experiments and provided technical help. XC provided comments and revised the manuscript. All authors have reviewed the manuscript and approved the final version.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

Animal studies were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals and the Principles for the Utilization and Care of Vertebrate Animals and approved by the Animal Research Committee of Sun Yat-sen University Cancer Center (SYSUCC) (Approval no. L102042020100L).

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41418-023-01242-w.

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