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ORIGINAL ARTICLE

A novel function of HRP-3 in regulating cell cycle progression via the HDAC–E2F1–Cyclin E pathway in lung cancer

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Abstract

To improve the poor survival rate of lung cancer patients, we investigated the role of HDGF-related protein 3 (HRP-3) as a potential biomarker for lung cancer. The expression of endogenous HRP-3 in human lung cancer tissues and xenograft tumor models is indicative of its clinical relevance in lung cancer. Additionally, we demonstrated that HRP-3 directly binds to the E2F1 promoter on chromatin. Interestingly, HRP-3 depletion in A549 cells impedes the binding of HRP-3 to the E2F1 promoter; this in turn hampers the interaction between Histone H3/H4 and HDAC1/2 on the E2F1 promoter, while concomitantly inducing Histone H3/H4 acetylation around the E2F1 promoter. The enhanced Histone H3/H4 acetylation on the E2F1 promoter through HRP-3 depletion increases the transcription level of E2F1. Furthermore, the increased E2F1 transcription levels lead to the enhanced transcription of *Cyclin E*, known as the E2F1-responsive gene, thus inducing S-phase accumulation. Therefore, our study provides evidence for the utility of HRP-3 as a biomarker for the prognosis and treatment of lung cancer. Furthermore, we delineated the capacity of HRP-3 to regulate the E2F1 transcription level via histone deacetylation.

KEYWORDS

Cyclin E, E2F1, HDACs, HRP-3, S-phase accumulation

1 | INTRODUCTION

Hepatoma-derived growth factor (HDFG)-related proteins (HRPs), including HDGF and HRP-3, are capable of binding to DNA. This is due to the presence of a PWWP domain (Pro-Trp-Trp-Pro residues are preserved) that can bind to DNA regardless of specificity.[1-4] The PWWP domain is known to be involved in DNA binding, transcriptional regulation processes, DNA repair, and chromatin remodeling through histone modification.[1] For instance, HDGF has been demonstrated to act as a transcriptional repressor. HDGF binds specifically to a conserved DNA element in the promoter of target genes such as *SET and MYND domain containing 1* (*SMYD1*) via its PWWP domain.[5]

Abbreviations: APAF1, apoptotic peptidase activating factor 1; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; cIAP-1, inhibitor of apoptosis protein 1; E2F1, E2F transcription factor 1; EPP, E2F1 promoter primer; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDGF, hepatoma-derived growth factor; HRP-3, HDGF-related protein 3; NSCLC, non–small cell lung cancer; PLA, proximity ligation assay; PWWP, Pro-Trp-Trp-Pro; Rb, retinoblastoma protein; SCLC, small cell lung cancer; SMYD1, SET and MYND domain containing 1; TSA, trichostatin A.

HRP-2 influences several biological functions including chromatin remodeling and DNA repair.[6] In addition, lens epithelium–derived growth factor, an HRP-2 protein, has been shown to function as a transcriptional coactivator.[7] Therefore, HRP-3, an HRP with a PWWP domain, is anticipated to act as a transcriptional regulator of specific genes through histone modification processes such as deacetylation of the promoter of specific genes. Furthermore, histone acetylation is another histone modification process, which is linked to cellular functions such as DNA replication and repair and chromatin assembly.[8]

Currently, the transcriptional function of E2F transcription factor 1 (E2F1) can be characterized in two ways. First, among the six E2F subfamilies, E2F1 was unique in its ability to induce apoptosis[9,10] and is activated transcriptionally by an apoptotic signal.[11,12] Second, E2F1 is an important regulator of the G1/S-phase in the cell cycle and is involved in cell growth regulation. During the cell cycle sequence, retinoblastoma protein (Rb) binds to E2F1 and rigidly regulates the functions of E2F1.[13,14] The phosphorylation of Rb by cyclin-dependent kinase (CDK) causes the release of E2F1 from Rb, which stimulates the transcription of E2F1 or Cyclin E. Cyclin E is an E2F1 transcriptional target that regulates DNA synthesis and activates entry into the S-phase.[15-18] Additionally, pRB binding to histone deacetylase 1-3 (HDAC1-3) suppresses the transcription of *E2F1*, or a gene that contains an E2F1 binding site.[19,20] The regulation of intracellular histone deacetylation and acetylation through HDACs and histone acetyltransferases (HATs), respectively, controls *E2F1* transcription levels.[21] The expression of *Cyclin E*, known as the E2F1-reponsive gene, is upregulated by E2F1 during the cell cycle, additionally functioning as an activator of CDK2. Furthermore, it accumulates at the G1/S boundary of the cell cycle and promotes functions associated with cell cycle entry into, and progression through, the S-phase.[22] High-level Cyclin E expression seems to be specific for tumor cells and may represent a potential prognostic biomarker of breast cancer.[23-25] Conversely, Cyclin E is regulated by genotoxic stress, and when activated by genotoxic stress, it plays a functional role in cell apoptosis.[26] Lastly, cells overexpressing Cyclin E exhibit prominent S-phase accumulation.[27]

Herein, we examined the regulatory functions of HRP-3 acting on E2F1 transcription by depleting HRP-3 in A549 cells. HRP-3 depletion led to increased E2F1 transcription and protein levels and induced increased S-phase accumulation via Cyclin E. Interestingly, it was determined that HRP-3 regulates the E2F1 transcription level and recruits HDAC1 and 2 to the E2F1 promoter. It was also observed that HRP-3 bound to the E2F1 promoter interacts with HDAC1 and 2, and this interaction results in histone modification. Herein, we elucidated the novel role of HRP-3 as a potential E2F1 transcriptional regulator by deacetylation of Histone H3 and H4 on the E2F1 promoter in suitable in vitro and in vivo models.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

Human lung cancer cell lines (H460 [HTB-177], A549 [CRM-CCL-185], and H1299 [CRL-5803]) were purchased from ATCC. H460, A549, and H1299 cells were cultured in RPMI-1640 medium. In all cases, media were supplemented with 10% fetal bovine serum.

2.2 | siRNA transfection

The following siRNA oligonucleotides targeting *HRP-3*,[28,29] *E2F1*, and *Cyclin E* were synthesized by Integrated DNA Technologies Inc: siHRP-3, 5′-GGCCAUGUGUAAAGUUUAAUU-3′; siE2F1, 5′-GUCACGCUAUGAGACCUCACUG-3′; and siCyclin E, 5′-AAGU GCUACUGCCGCAGUAUCC-3′. The siRNA duplexes were transfected into cells using Metafectene reagent (Biontex) according to the manufacturer's guidelines.

2.3 | Western blot analysis

Western blot analyses were performed as previously described[30] using primary antibodies targeting the following proteins: HRP-3 (Proteintech group [12380-1-AP]); HRP-3 (Novus Biologicals [NBP2-13246]); E2F1 (Sc-137059), Cyclin E (sc-48420), HDAC1 (sc-81598), HDAC2 (sc-9959), α-tubulin (sc-5289), PCNA (sc-25280), and GFP (sc-9996) (Santa Cruz Biotechnology); and acetylated Histone H3 (06-599) and H4 (06-866) or nonacetylated Histone H3 (06-755) and H4 (07-108) (Millipore). β-actin (Sigma Aldrich [A5441]) was used as a loading control.

2.4 | Flow cytometer (FACS) analysis for synchronizing at G1/S-phase of cells and analysis of S-phase accumulation

Cells were subjected to the double thymidine block/release block protocol,[31] and synchronizing at the G1/S-phase was analyzed with a FACScan flow cytometer (BD Biosciences). To analyze S-phase accumulation by FACS, 2 hours before the endpoint (48 hours post siRNA transfection), EdU from the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Cat. C10420; Thermo Fisher Scientific) was added to the cell culture medium to a final concentration of 10 μM. Cells were rinsed twice with PBS, trypsinized, fixed/permeabilized, and the Click-iT EdU Alexa Fluor 488 reaction was performed according to the manufacturer's instructions. To avoid compensation, DNA was stained for 30 minutes at room temperature using FxCycle Far Red fluorescence (Cat. F10348; Thermo Fisher Scientific).

2.5 | Chromatin-binding assay

The method previously described by He et al[32] was used, with the modifications described by Kannouche et al.[33] Briefly, 36 hours after transfection, cells were lysed using cold CSK (Cytoskeletal) I buffer (10 mmol/L PIPES [pH 6.8], 100 mmol/L NaCl, 1 mmol/L EDTA, 300 mmol/L sucrose, 1 mmol/L $MgCl_2$, 1 mmol/L DTT) supplemented with 0.5% (v/v) Triton X-100, protease inhibitors (Roche

Complete Mini), and 1 mmol/L phenylmethylsulfonyl fluoride; the cell suspension was incubated on ice for 15 minutes. One-tenth of the lysate (total fraction, T) was mixed with RIPA buffer (150 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 0.5% DOC (Sodium deoxycholate), 0.1% [w/v] SDS, 1% [v/v] NP-40). The remaining cell lysate was divided into two equal portions, which were centrifuged at 500 *g* at 4°C for 3 minutes. The supernatants (S1 fraction), which contained the triton-soluble proteins, were analyzed. One of the pellets, which contained chromatin-bound, nuclear matrix–bound, and insoluble proteins, was resuspended in RIPA buffer (the P1 fraction). The other pellet was resuspended in CSK (Cytoskeletal) II buffer (10 mmol/L PIPES [pH 6.8], 50 mmol/L NaCl, 300 mmol/L sucrose, 6 mmol/L $MgCl_2$, 1 mmol/L DTT), treated with DNase for 30 minutes, followed by extraction with 250 mmol/L NH_2SO_4 for 10 minutes at 25°C. The sample was treated with DNase and salt and centrifuged at 1200 *g* for 6 minutes at 4°C, and the supernatant (S2 fraction, containing DNase-released chromatin-associated proteins) and pellet (P2, containing insoluble, cytoskeletal, and nuclear matrix proteins) were collected. P2 was resuspended in RIPA buffer. All fractions were analyzed using immunoblotting and Western blotting.

2.6 | Xenograft tumor animal model using A549 cells

Studies with the tumor xenograft animal model were performed as previously described[34] using siHRP-3.[28,29] Animal handling was conducted in accordance with the protocol approved by the Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences (KIRAMS 2015-0070).

Detailed methods for plasmid construction and transfection, quantitative reverse-transcription–polymerase chain reaction (qRT-PCR), reporter gene assay, in situ proximity ligation assay (PLA), immunohistochemistry, immunofluorescence confocal microscopy, chromatin immunoprecipitation (ChIP) and reChIP assay, data mining using Kaplan-Meier plotter, and statistical analysis are described in Appendix S1.

3 | RESULTS

3.1 | HRP-3 found to be associated with poor clinical outcomes in lung cancer patients and tumor growth in the xenograft tumor model

In a previous study, we identified the ability of HRP-3 to induce death in lung cancer cells.[28,29] Herein, we first investigated the HRP-3 expression levels in lung cancer patients. Notably, in 45 of 59 lung cancer patients, HRP-3 was upregulated in lung cancer tissue compared with the level observed in the corresponding normal tissue (Figure 1A), and the average HRP-3 staining score in cancer tissues was significantly higher than that in the paired adjacent normal tissues (Figure 1B). Additionally, in a previous study conducted, Kaplan-Meier plotter analysis revealed an inverse correlation between HRP-3 expression level in tumor samples from lung cancer patients and poor survival[35] (Figure 1C). Furthermore, patients exhibiting a high expression level of HRP-3 showed poor prognosis after radiation and chemotherapy (Figure S1). Next, we determined the clinical applicability of the xenograft tumor model using siHRP-3. As shown in Figure 1D, tumor volumes of the siHRP-3 group were significantly reduced compared with those in the scrambled group. After termination of the experiment, tumor images (Figure 1E) and tumor weight measurements (Figure 1F) of the siHRP-3 and scrambled groups were consistent with the tumor volume results and were supported by the results of immunostaining confocal fluorescence microscopy analysis of tumor tissue (Figure 1E, right panel). These data indicated that HRP-3 may serve as a key protein relevant for the treatment and prognosis of lung cancer patients.

3.2 | Effect of siHRP-3 on the E2F1–Cyclin E axis and S-phase accumulation

The unique function of HRP-3, which is a potential therapeutic target for lung cancer, is not well defined and needs to be studied. In A549 cells, the HRP-3 protein has the same location as DNA, and the amount of HRP-3 at the interphase reduces from early mitosis; however, cytokinesis induces an increase in its level (Figure 2A). The double thymidine block results of A549 cells and HRP-3 expression peaked in the S-phase and decreased during the G2/M-phase (Figure 2B). Therefore, HRP-3 is expected to play a unique role in the S-phase, and siHRP-3 was transfected into A549 cells to monitor the ensuing changes in the S-phase. HRP-3 depletion induced an increase in S-phase accumulation compared with the rate of accumulation in the scrambled group (Figure 2C). Cells overexpressing Cyclin E accumulate in the S-phase[36,37] and *Cyclin E* is the transcriptional target gene for E2F1.[38] The expression levels of HRP-3 and E2F1/Cyclin E had a negative association with each other in the interphase (6 hours after thymidine treatment), especially at the S-G2 transition phase, as observed using Western blotting of A549 cell lysates treated with a double thymidine block (Figure 2B). HRP-3 depletion augmented the transcription levels of E2F1 and Cyclin E at 48 hours (Figure 2D), as well as increased protein levels of E2F1 and Cyclin E (Figure 2E). In addition, reporter gene assay results using the human E2F1 promoter linked to luciferase showed that siHRP-3 increased the transcription level of E2F1 (Figure 2F). Next, A549 cells were transfected with either siHRP-3, siE2F1, or both; we observed that the elevated expression of Cyclin E induced by siHRP-3 was reduced by siHRP-3 combined with siE2F1 (Figures 2G,H and S2). Moreover, when A549 cells were transfected with siHRP-3, siE2F1, and siCyclin E, respectively, or all three simultaneously, the siHRP-induced S-phase accumulation was inhibited by transfection with siHRP-3 combined with siE2F1 or siCyclin E (Figure 2I). These results provided evidence that HRP-3 exerts a novel function in regulating S-phase progression through regulation of the E2F1 transcription level.

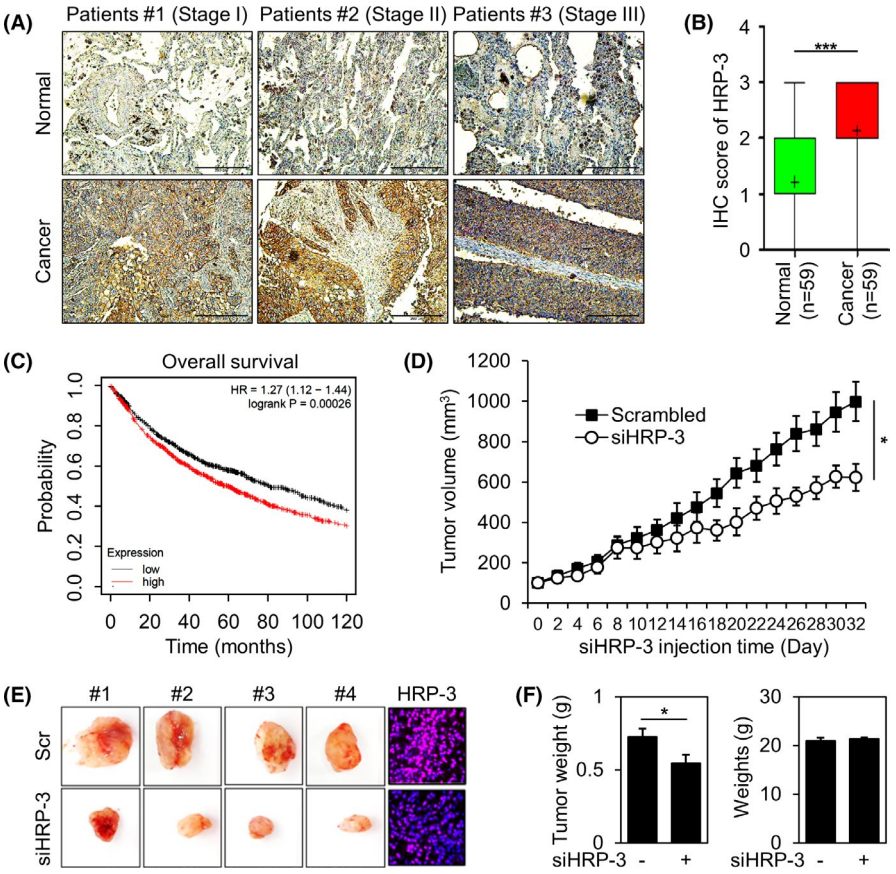

FIGURE 1 Importance of HRP-3 as a novel prognostic factor and therapeutic target in lung cancers. A, Microscopic images of lung cancer tissues and their normal tissue counterparts stained with HRP-3 antibody (scale bar: 50 μm). B, Staining intensity was scored as follows: 0, no staining; +1, weak; +2, moderate; and +3, strong. Data are presented as box-and-whisker plots. ***$P < .001$. C, Effects of HRP-3 on the overall survival of lung cancer patients, using Kaplan-Meier plotter analysis. D, Effects of HRP-3 depletion on non–radiation-induced tumor growth delay by subcutaneous injection of 1×10^6 A549 cells. The mice were subjected to tail vein injection every 2 d with scrambled (control siRNA) or siHRP-3 (40 μg siRNA/mouse) when the tumor volume reaches 100 mm^3. Tumor volume using scrambled and siHRP-3 was calculated at the indicated time points using the formula: volume = (length × $width^2$ × 3.14)/6; (n = 10). *$P < .05$. E, Tumors treated with Scr (scrambled) and siHRP-3 were excised and weighed at the end of the experiment (30 d). Images of tumors and tumor slides subjected to immunohistochemistry using HRP-3 antibody. F, Graph of tumor and body weight. *$P < .05$

3.3 | HRP-3 found to bind to the E2F1 promoter on chromatin

As HRP-3 silencing induced an increase in E2F1 mRNA expression, we investigated whether HRP-3 directly regulates E2F1. HRP-3 contains a PWWP domain that can bind both DNA and histones.[1,2] Furthermore, the protein levels of wild-type GFP-HRP-3 (GFP-HRP-3 [WT]) and the GFP–PWWP-deletion mutant variant of HRP-3 (GFP-HRP-3 [ΔPWWP]) were verified using Western blotting (Figure 3A,B). The chromatin-binding ability of HRP-3 was verified using a chromatin-binding assay; its intracellular distribution was established after identifying its intracellular location (Figure 3C). GFP-control does not interact with chromatin, and it has a pancellular distribution; it is confined to the Triton-soluble fraction (S1, not–chromatin-bound fraction). The cytosolic protein α-tubulin was found exclusively in S1, while the proliferating cell nuclear antigen (PCNA) was distributed in both Triton-soluble (S1) and Triton-resistant (P1, chromatin-bound fraction) fractions. PCNA was extracted efficiently with DNase and salt. Histone H3 was recovered only from the Triton-resistant fractions. It was extracted more efficiently with DNase and salt (S2, DNase-released chromatin-bound fraction) than with RIPA buffer (P1 and T). GFP-HRP-3 (WT) was strongly chromatin bound and remained exclusively in the Triton-insoluble fraction (P1), and it was released using S2. Additionally, we transfected A549 cells with GFP-HRP-3 (ΔPWWP); deletion of PWWP was found exclusively in S1. Following the analysis of these domains as individual segments in test fusion, we performed the complementary analysis to ascertain whether the PWWP domain is essential for the chromatin association in the context of wild-type HRP-3 (Figure 3D). GFP-control exhibited a diffuse nuclear and cytosolic distribution in the interphase, with a lack of interaction with condensed mitotic chromatin. GFP-HRP-3 (WT) exhibited both a fine speckled nuclear pattern during the interphase and binding to mitotic chromatin in A549 and H1299 cells. GFP-HRP-3 (ΔPWWP) was expressed stably in A549 and H1299 cells. It had a diffuse nuclear pattern in

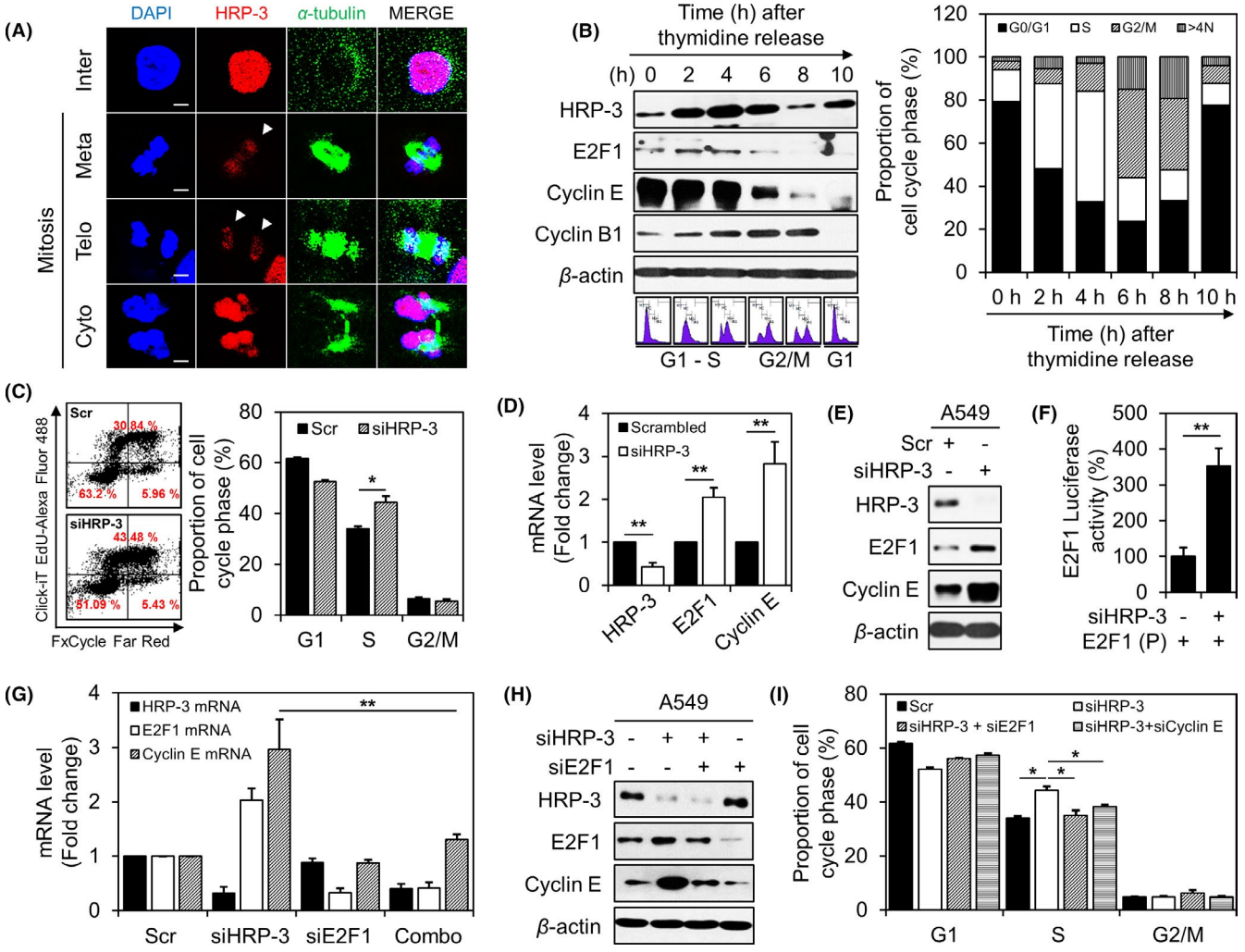

FIGURE 2 Depletion of HRP-3 promotes accumulation of abnormal S-phase via the E2F1Cyclin E axis. A, Expression levels of HRP-3 (red) and α-tubulin (green) protein were determined using confocal microscopy. Cells were identified by DAPI (blue) staining of nuclei. B, A549 cells were synchronized by a double thymidine block released into fresh medium at the indicated time points and analyzed by immunoblotting with antibodies against the indicated proteins (left). Levels of HRP-3, E2F1, and Cyclin E detected by Western blotting. E2F1 and Cyclin E were used as positive controls for the interphase. Graphs representing mean percentages of G0/G1-, S, G2/M-phase, and >4N cell populations synchronized using a double thymidine block are obtained from cell cycle analysis using a FACScan flow cytometer (right). C, S-phase accumulation analysis of A549 cells (transfected with 50 nmol/L scrambled or siHRP-3) using a FACScan flow cytometer. $^{*}P < .05$. D-E, A549 cells were transfected with 50 nmol/L scrambled and siHRP-3 and harvested. The mRNA levels and protein levels of HRP-3, E2F1, and Cyclin E were determined by qRT-PCR and Western blotting. $^{**}P < .01$. F, A549 cells were cotransfected with 50 nmol/L siHRP-3 and 1 μg control constructs or constructs containing the human E2F1 promoter for 24 h. E2F1 promoter transcriptional activity was assessed using a luciferase reporter gene assay. $^{**}P < .01$. G-H, A549 cells were transfected with either 50 nmol/L of siHRP-3, 50 nmol/L of siE2F1, or both. The mRNA levels and protein levels of HRP-3, E2F1, and Cyclin E were determined by qRT-PCR and Western blotting. $^{**}P < .01$. I, S-phase accumulation analysis in A549 cells transfected with either 50 nmol/L siHRP-3, 50 nmol/L siE2F1, 50 nmol/L siCyclin E, or all three of them using a FACScan flow cytometer. $^{*}P < .05$

the interphase, with a lack of interaction with condensed mitotic chromatin. Therefore, our biochemical results indicated that the PWWP domain of HRP-3 is a major determinant of the binding capacity of HRP-3 to the chromatin of NSCLC cell lines, such as A549 cells. Next, to evaluate whether HRP-3 binds to the E2F1 promoter, a ChIP assay was performed using HRP-3 antibody in A549 cells. As shown in Figure 4A, we used five PCR primers (*E2F1* promoter primer [EPP]; #1-#5) based on the wild-type human *E2F1* promoter sequence (Figure 4A, upper). While we observed quantitative differences in the PCR amplicons of the five primers, the HRP-3 antibody precipitated *E2F1* promoter DNA (Figure 4A, bottom). Next, we carried out a ChIP assay using A549 cells depleted of HRP-3. ChIP results revealed that the amount of PCR product amplified using EPP#5 (included E2F1 binding site) was reduced in HRP-3–depleted A549 cells compared with the amount of amplified product in control A549 cells, in accordance with the concentration gradient of siHRP-3 (Figure 4B). Thus, these data indicated that HRP-3 directly binds to the *E2F1* promoter in A549

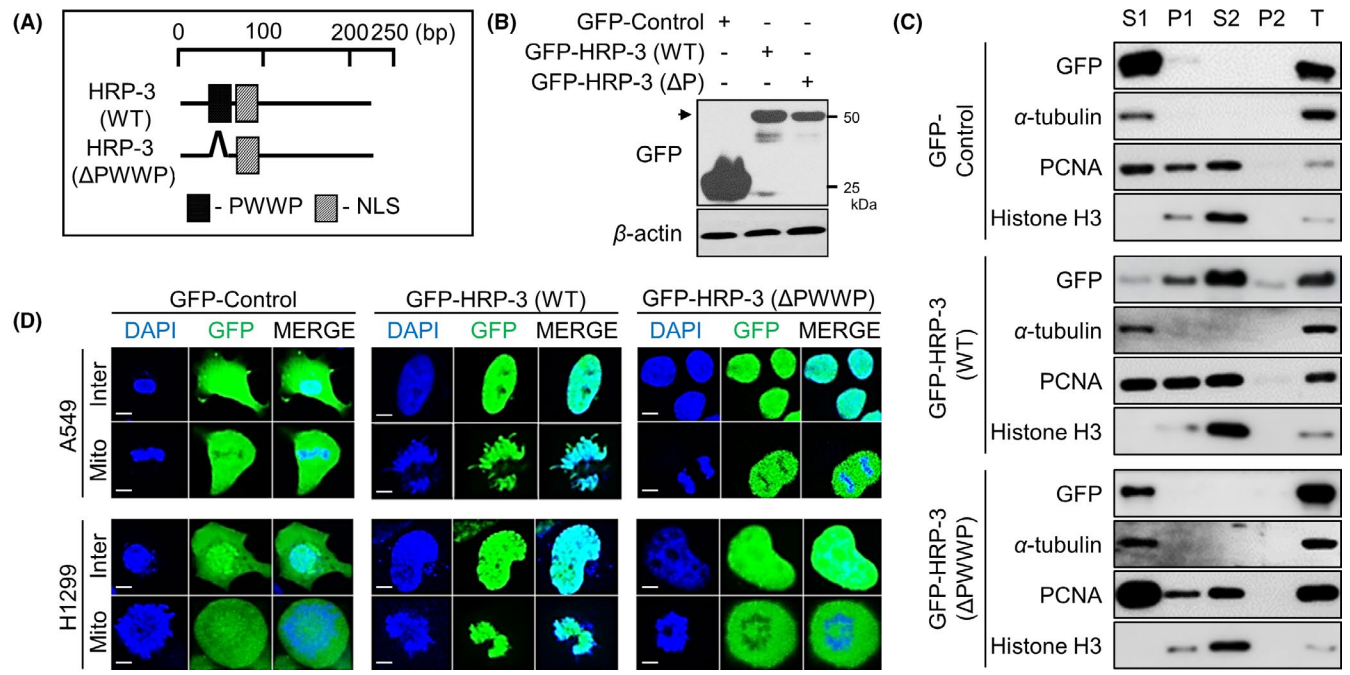

FIGURE 3 Transcription factor HRP-3 binds to the DNA of lung cancer cells by relying on the PWWP domain. A, HRP-3 domain structure and a schematic representation of the HRP-3 hybrid. Amino acid sequential numbering from the N-terminus: PWWP, 20-26; NLS, 70-80 (left). B, Western blot analyses of wild-type GFP-HRP-3 (GFP-HRP-3 [WT]) and the GFP–PWWP-deletion mutant variant of HRP-3 (GFP-HRP-3 [ΔPWWP]) in transfected A549 cells. C, Fractionation patterns of GFP-HRP-3 (WT) and GFP-HRP-3 (ΔPWWP) in a chromatin-binding assay (S1: triton soluble proteins; P1: chromatin-bound, nuclear matrix-bound, and insoluble proteins; S2: DNase-released chromatin-associated proteins; P2: insoluble, cytoskeletal, and nuclear matrix proteins; T: total protein extract). Fractionation patterns of control proteins (GFP, α-tubulin, proliferating cell nuclear antigen [PCNA], Histone H3) were evaluated using Western blot analysis. D, Confocal microscopy observation of A549 and H1299 cells transfected with GFP-control, GFP-HRP-3 (WT), and GFP-HRP-3 (ΔPWWP)

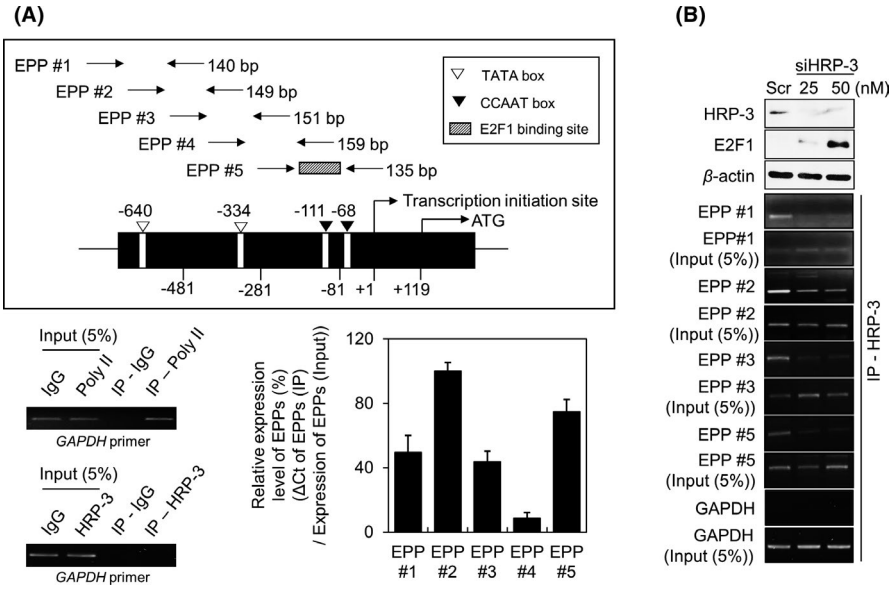

FIGURE 4 Binding of HRP-3 to the E2F1 promoter in the chromatin of lung cancer cells. A, Schematic diagram of the human E2F1 promoter and the primer set used for the ChIP assay (upper). The coprecipitated chromatin DNA was analyzed using qRT-PCR with a pair of E2F1 promoter primer (EPP) that amplified the −640 to −81 bp region on the E2F1 promoter. ChIP assay was performed using sheared chromatin from formaldehyde–cross-linked A549 cells using anti-HRP-3 as the immunoprecipitating antibody (bottom). Control primers specific for human *GAPDH* and normal mouse IgG and anti-RNA polymerase II as the immunoprecipitating antibody were used as negative and positive control, respectively. B, A549 cells transfected with 25 and 50 nmol/L siHRP-3 were incubated for 48 h. Protein levels of HRP-3 and E2F1 were determined by Western blotting (upper) and ChIP assay was performed using sheared chromatin from formaldehyde–cross-linked A549 cells using anti-HRP-3 as the immunoprecipitating antibody (bottom)

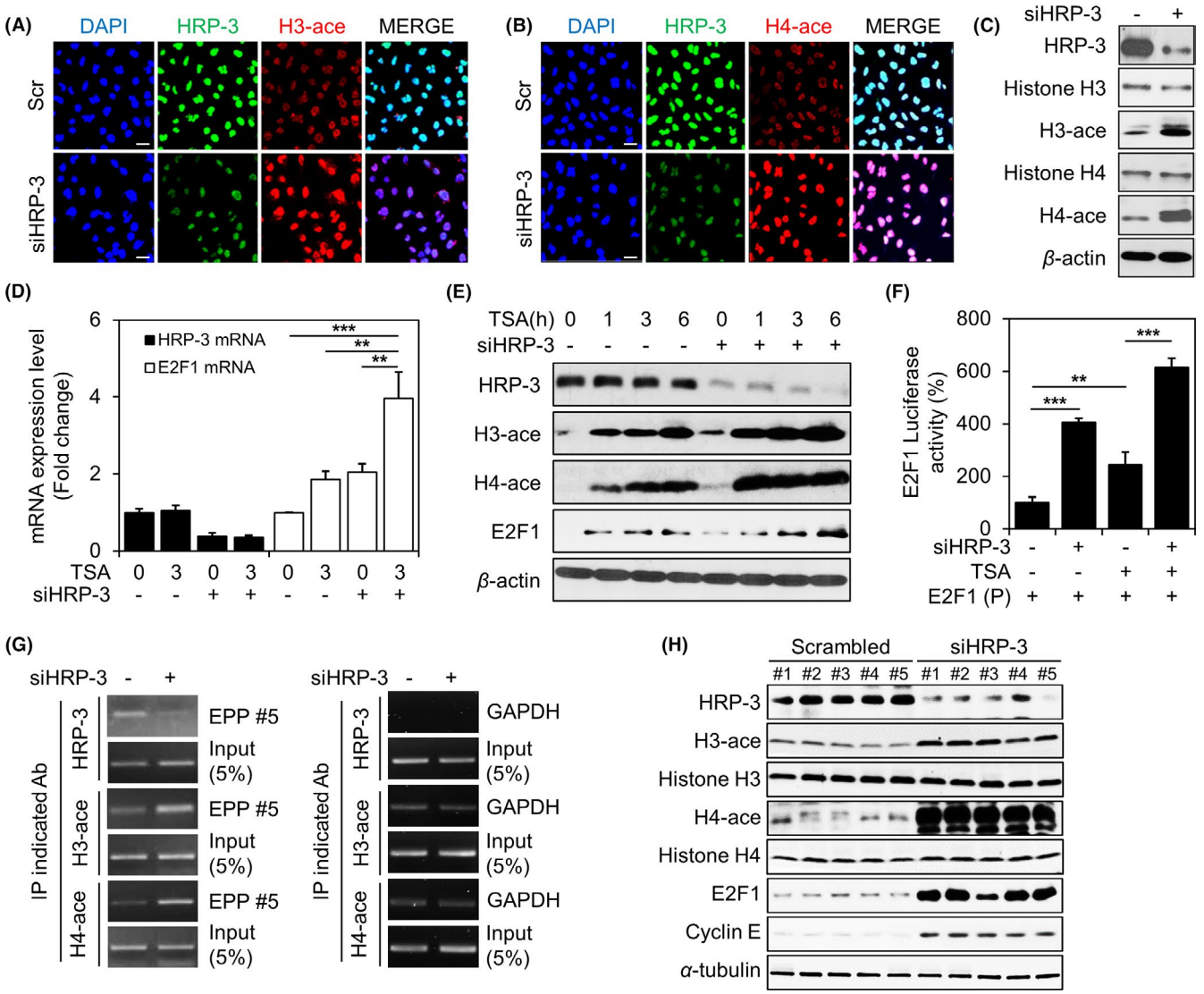

FIGURE 5 Effect of HRP-3 depletion on the regulation of E2F1 expression in lung cancer cells. A-B, HRP-3 siRNA-treated cells were stained with the indicated proteins and imaged by confocal microscopy. C, A549 cells were transfected with 50 nmol/L scrambled or siHRP-3 for 48 h. Protein levels of the indicated proteins were determined by Western blotting. D-E, HRP-3–deficient A549 cells transfected with 50 nmol/L siHRP-3 were treated with 100 nmol/L TSA for the indicated periods of time. The mRNA levels of *E2F1* were detected by qRT-PCR (D), and the protein levels of the indicated proteins were determined by Western blotting (E). $^{**}P < .01$; $^{***}P < .001$. F, A549 cells were cotransfected with 50 nmol/L siHRP-3 and 1 μg control constructs or constructs containing the human E2F1 promoter for 24 h, and then treated with 50 μmol/L TSA for 3 h. E2F1 promoter transcriptional activity was assessed using a luciferase reporter gene assay. $^{**}P < .01$; $^{***}P < .001$. G, A549 cells were transfected with 50 nmol/L scrambled or siHRP-3 and cultured for 48 h. ChIP assay was performed using sheared chromatin from formaldehyde–cross-linked A549 cells using anti-HRP-3 and antiacetylation of Histone H3 and H4 as the immunoprecipitating antibodies. The GAPDH promoter primer was used as a negative control. H, Western blot analysis was performed to determine protein levels of the indicated proteins in xenograft tumors

cells regardless of the DNA sequence and implied that HRP-3 binding to the *E2F1* promoter may regulate *E2F1* transcription.

3.4 | HRP-3 depletion induced histone modifications, which regulate E2F1 transcriptional levels

Transcription is regulated through epigenetic modifications of histones, such as acetylation and deacetylation.[39] Histone acetylation via trichostatin A (TSA), which is capable of inhibiting HDAC class I and II, enhances the transcription level of E2F1.[21] Immunostaining confocal fluorescence microscopy and Western blot analyses showed that transfection with siHRP-3 in A549 cells increased the levels of the acetylated form of Histone H3 and H4 compared with the corresponding levels in A549 cells transfected with a control siRNA (Figure 5A-C). Interestingly, when A549 cells were treated with TSA after HRP-3 depletion, the E2F1 transcription level increased synergistically compared with the corresponding level observed in the control (Figure 5D). These results are consistent with the Western

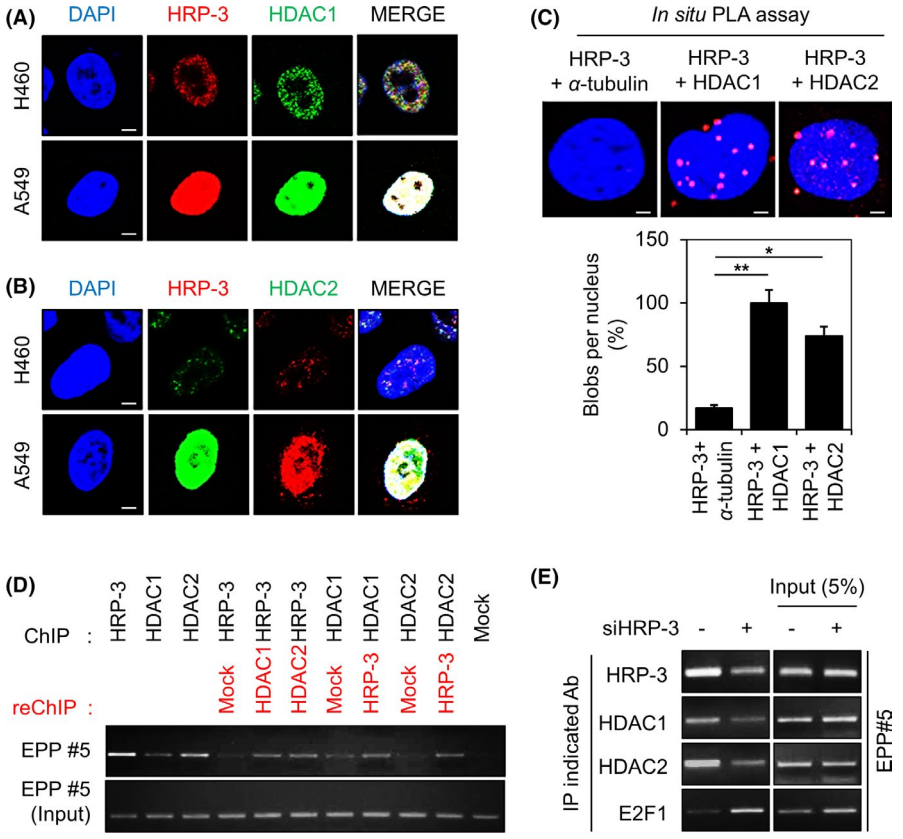

FIGURE 6 Interaction between HRP-3 and HDAC1/2 on the E2F1 promoter of lung cancer cells. A-B, Expression levels of HRP-3 (red/green) and HDAC1 (green)/HDAC2 (red) proteins were determined using confocal microscopy. C, A549 cells were fixed and incubated with mouse or rabbit anti-HRP-3 together with mouse anti-HDAC1 or rabbit-HDAC2, followed by in situ proximity ligation assay (PLA) analysis. Confocal images of cells with PLA-positive signals are shown (red dots). Interaction between anti-HRP-3 and anti-α-tubulin was used as a negative control. $^{*}P < .05$; $^{**}P < .01$. D, reChIP assay was performed after an initial immunoprecipitation with anti-HRP-3 and anti-HDAC1/2 antibodies in A549 cells. A protein-DNA complex was recovered after a second immunoprecipitation with HRP-3 and HDAC1/2 antibodies. E, A549 cells were transfected with 50 nmol/L scrambled or siHRP-3 and cultured for 48 h. ChIP assay was performed using sheared chromatin from formaldehyde–cross-linked A549 cells using anti-HRP-3, anti-HDAC1, anti-HDAC2, and anti-E2F1 as the immunoprecipitating antibodies

blot results of Histone H3 and H4 acetylation (Figure 5E). Next, we carried out an *E2F1* promoter assay using siHRP-3 combined with TSA. While the *E2F1* transcriptional activity was increased by HRP-3 depletion, it was further elevated by the synergistic effect of HRP-3 depletion combined with TSA (Figure 5F). Additionally, HRP-3 depletion in A549 cells induced acetylation of Histone H3 and H4 on the *E2F1* promoter (Figure 5G). The tumor tissues from siHRP-3–treated xenografts exhibited increased acetylation of Histone H3 and H4 and increased protein levels of E2F1 and Cyclin E (Figure 5H). Therefore, these findings indicated that HRP-3 depletion in A549 cells induces acetylation of Histone H3 and H4 in vitro and in vivo, in addition to modulating E2F1 promoter activity.

3.5 | Recruitment of HDAC1/2 to the E2F1 promoter by HRP-3

We hypothesized that HRP-3 without a catalytic domain[4,40] would regulate acetylation of H3 and H4 by interacting with the HDAC family as a molecular partner on the *E2F1* promoter. The HDAC1/2 protein level was increased in the A549 cell line compared with the corresponding level in the H460 cell line (Figure 6A,B). Interestingly, immunostaining confocal fluorescence microscopy analysis revealed that HRP-3, HDAC1, and HDAC2 localize to the nucleus; additionally, HRP-3, HDAC1, and HDAC2 signals exhibited significant degrees of overlap in both H460 and A549 cells (Figure 6A,B). Next, to confirm the interaction between HRP-3 and HDAC1/2 in the nucleus, we carried out an in situ proximity ligation assay (in situ PLA) in A549 cells. Positive signals (red dots) indicated interaction between HRP-3 and HDAC1/2 proteins in the nucleus (Figure 6C). A549 cells transfected with siHRP-3 exhibited a reduced interaction between HRP-3 and HDAC1/2 proteins (Figure S3). Finally, to determine whether HRP-3 interacts with HDAC1/2 on the *E2F1* promoter, we carried out a reChIP assay of HRP-3, HDAC1, and HDAC2. The reChIP assay showed that endogenous HRP-3, HDAC1, and HDAC2 are localized on the *E2F1* promoter; moreover, the binding of HDAC1 and 2 to the *E2F1* promoter was determined to be mediated by HRP-3 (Figure 6D). Additionally, HRP-3 depletion weakened the binding of HDAC1 and HDAC2 to the *E2F1* promoter, while enhancing the binding of E2F1 (Figure 6E). These results indicated that HDAC1 and

2 recruited by HRP-3 to the *E2F1* promoter regulate the acetylation and deacetylation of Histone H3 and H4 around the *E2F1* promoter, thus modulating *E2F1* transcription.

4 | DISCUSSION

Lung cancer is a carcinoma with a high mortality rate worldwide and can be clinically divided into small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC). Approximately 80%-85% of lung cancer patients are diagnosed with NSCLC.[41] Advanced stages of NSCLC, compared with stage I NSCLC, manifest low survival rates due to its inherent resistance to radiotherapy or chemotherapy.[42] Therefore, to achieve a superior lung cancer therapeutic outcome, we sought for a better understanding of HRP-3 as a target molecule in human lung cancer treatment. We previously reported that the depletion of HRP-3 in A549 and H1299 cells leads to reactive oxygen-dependent cell death caused by radiation.[28,29] However, as our study focused on the link between the HRP-3 expression level and radioresistance in each NSCLC cell line, at the time we could not clearly elucidate the trend of HRP-3 expression levels in lung cancer patients. Interestingly, HRP-3 expression is higher in cancer tissues than in normal tissues and the prognosis of patients with elevated HRP-3 expression is poor. Furthermore, HRP-3 expression impacts the prognosis of radiation and anticancer treatment, and HRP-3 depletion in a xenograft tumor model demonstrated a favorable outcome for tumor treatment (Figures 1 and S1). These findings carry considerable clinical relevance, implying that HRP-3 may be utilized as a biomarker indicative of the prognosis of patients and as a new target lung cancer treatment agent.

In the current study, HRP-3 was shown to induce proliferative arrest in Granta 519 MCL cells[43] and regulate invasion migration of hepatocellular carcinoma[44] as a mitogen factor; however, the association between HRP-3 and the cell cycle has not been completely elucidated. Throughout the cell cycle, HRP-3 colocalized with DNA, increased in the G1-phase, and peaked in the S-phase followed by a decrease during G2/mitosis. Inhibition of HRP-3 expression causes abnormal accumulation of the S-phase; therefore, HRP-3 is involved in S-phase progression. As the HDGF family, to which HRP-3 belongs, is known to act as a transcription repressor,[45] we investigated whether HRP-3 functions as a transcription repressor in the S-phase. In the cell cycle, E2F1 is a transcription factor that regulates the G1/S-phase transition by inducing the expression of genes such as *Cyclin E*.[46] Following G1/S transition, E2F1 is ubiquitinated and degraded by inhibitor of apoptosis protein 1 (cIAP-1) in the S-phase.[46] We demonstrated that HRP-3 depletion in A549 cells increased endogenous E2F1 expression levels, thereby enhancing the transcriptional activity of Cyclin E. The coknockdown of HRP-3 and E2F1 hindered the increase in the Cyclin E expression level generated by HRP-3 depletion, thus recovering the abnormally accumulated S-phase. This observation indicated that HRP-3 may play an important role in cell cycle progression.

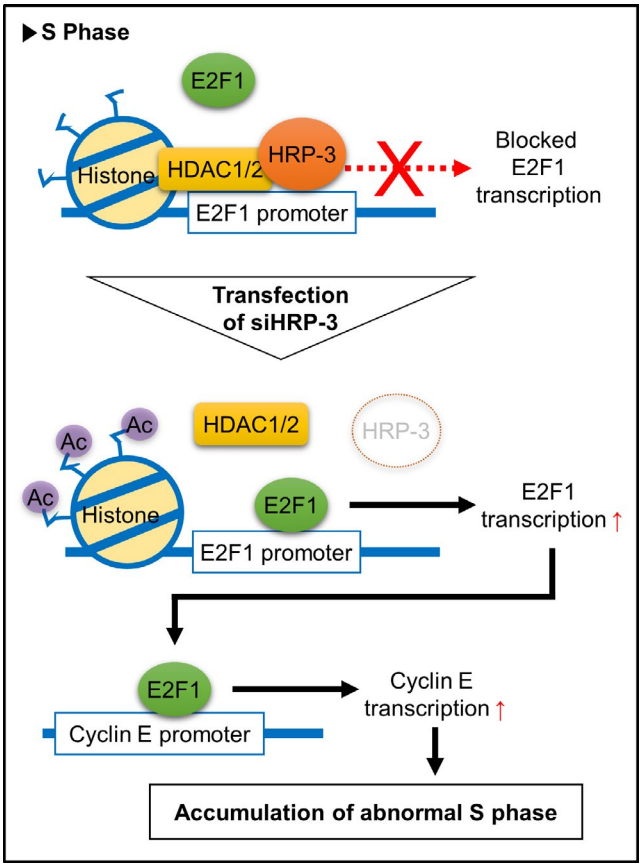

FIGURE 7 A schematic representation of the E2F1 transcription level regulated by the interaction between HRP-3 and HDAC 1/2 and the associated cell cycle changes

The HDGF family of proteins, including HRP-3, exhibit a PWWP domain capable of binding to chromatin nonspecifically, and proteins with the PWWP domain possess chromatin-related functions such as DNA repair and histone modification.[1-4] We demonstrated that HRP-3 binds to the entire chromatin content of lung cancer cells through its PWWP domain, which is in line with the results reported by Wei Tian et al in liver cancer cells.[47] Also, HDGF has been shown to bind to a distinct DNA motif and function as a negative transcriptional regulator for different target genes.[45] Herein, we showed that HRP-3 binds to various sites, including the E2F1 binding site on the E2F1 promoter. In addition, HRP-3 depletion facilitates E2F1 promoter activity. Based on these results, we hypothesized that the transcriptional regulation of E2F1 by HRP-3 may be linked to the E2F1 binding site. We also demonstrated that the interaction between HRP-3 and HDAC1/2 regulates Histone H3 and H4 modification on the E2F1 promoter, thus modulating the transcription level of E2F1. Anne-Laurence et al[21] showed an increase in E2F1 and E2F1-responsive genes (*Cyclin E* and *apoptotic peptidase activating factor 1 [APAF1]*), while inducing acetylation of Histone H3 and H4 by treating granule neurons obtained from mice with TSA (HDAC class I and II inhibitor). Our study showed that HRP-3 depletion induces acetylation of Histone H3 and H4 in the E2F1 promoter,

which is caused by the abrogation of the recruitment of HDAC1 and 2 by HRP-3. This allows the recruitment of the E2F1 protein to the E2F1 promoter (Figure 7). The acetylation of Histone H3 and H4, in vitro and in vivo by HRP-3 depletion, could potentially regulate the transcription of other genes in addition to that of E2F1. Therefore, accurate assessment using ChIP sequencing (ChIP-seq) to identify acetylation of Histone H3 and H4 on the promoters of various genes in HRP-3–depleted cells is essential. In addition, the transcription levels of E2F1 and E2F1-responsive genes are inhibited by the pRb-HDAC complex (HDAC1-3).[48,49] However, HRP-3 could modulate the degree of E2F1 transcription by interfering with the E2F1 promoter, independent of the pRb protein. Furthermore, prohibitin, associated with transcriptional inhibition of E2F1, binds to other E2F1 promoter regions; this indicates that prohibitin-induced E2F1 inhibition could be mediated by signals that do not target the Rb protein.[50] Therefore, further research is warranted to clarify whether the regulation of E2F1 transcription by HRP-3 is dependent or independent of pRb protein.

Our study shows that HRP-3 is both a potential indicator of the development and progression of various cancers and an indicator of the clinical outcome of radio-chemotherapy. Furthermore, we provide new molecular insights into E2F1 transcription regulation by HRP-3- and HDAC-induced histone modifications.

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DISCLOSURE

All authors declared that they had no conflict of interest.

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