Regulation of miRNA Biogenesis and Histone Modification by K63-Polyubiquitinated DDX17 Controls Cancer Stem-like Features

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Abstract

Markers of cancer stemness predispose patients to tumor aggressiveness, drug and immunotherapy resistance, relapse, and metastasis. DDX17 is a cofactor of the Drosha–DGCR8 complex in miRNA biogenesis and transcriptional coactivator and has been associated with cancer stem-like properties. However, the precise mechanism by which DDX17 controls cancer stem-like features remains elusive. Here, we show that the E3 ligase HectH9 mediated K63-polyubiquitination of DDX17 under hypoxia to control stem-like properties and tumor-initiating capabilities. Polyubiquitinated DDX17 dissociated from the Drosha–DGCR8 complex, leading to decreased biogenesis of anti-stemness miRNAs. Increased association of polyubiquitinated DDX17 with p300–YAP resulted in histone 3 lysine 56 (H3K56) acetylation proximal to stemness-related genes and their subsequent transcriptional activation. High expression of HectH9 and six stemness-related genes (BMI1, SOX2, OCT4, NANOG, NOTCH1, and NOTCH2) predicted poor survival in patients with head and neck squamous cell carcinoma and lung adenocarcinoma. Our findings demonstrate that concerted regulation of miRNA biogenesis and histone modifications through posttranslational modification of DDX17 underlies many cancer stem-like features. Inhibition of DDX17 ubiquitination may serve as a new therapeutic venue for cancer treatment.

Significance: Hypoxia-induced polyubiquitination of DDX17 controls its dissociation from the pri-miRNA–Drosha–DGCR8 complex to reduce anti-stemness miRNA biogenesis and association with YAP and p300 to enhance transcription of stemness-related genes.

Introduction

Cancer stem-like cells (CSC) possess the ability to self-renew and differentiate into mature tumor cells inside a tumor mass. These stem-like features of CSCs are associated with higher tumorigenicity, drug response, relapse, and metastasis in patients with cancer (1). Recently, stemness features, which were extracted from transcriptomic and epigenetic signatures and identified by machine learning algorithm, have also shown correlation with immunotherapy response (2). Understanding the mechanisms leading to stemness is thus vital for treating cancers.

Among the proteins that regulate pluripotency and reprogramming are the DEAD-box RNA-binding proteins (RBPs; ref. 3). DEAD-box RBPs have critical roles in RNA metabolic pathways, including transcription, splicing, miRNA biogenesis, translation, and decay (4). One important DEAD-box RBP is DDX17, which is a known cofactor of the Drosha Microprocessor and facilitates recognition and processing of primary miRNAs (pri-miRNAs) to become mature miRNAs (5). The Microprocessor is mainly constituted of Drosha (a ribonuclease) and DGCR8 (a double-stranded RNA binding protein; ref. 6). However, different cofactors, such as DDX5 and DDX17, hnrNP A1, and BRCA1 can associate with the Microprocessor to modulate its activity (7–10). Depending on their downstream targets, miRNAs may function as oncogenes or tumor suppressor genes (11). In human cancers, sequestration of DDX17 by YAP in the Hippo pathway has been reported to cause global downregulation of miRNAs (9). DDX17 also acts as a transcriptional coactivator for a range of transcription factors, which cause tumorigenicity in cancers (12, 13).

Interestingly, DDX17 and other RBPs can be regulated by posttranslational modifications (PTM), which can change RNA-binding affinity, function, and localization, thereby contributing additional layers of regulatory complexity (14). PTMs in the form of ubiquitination via K63 linkage function as a platform for protein–protein interactions (14). HectH9 is one of the few E3 ligases that can trigger both K63- and K48-linked ubiquitination (15) and has been implicated in the maintenance and development of stem cells (16) and tumor progression (17).
HectH9 is overexpressed in various cancers (17) and has been reported to be a potential target for anticancer therapies (18). In locally advanced solid tumors, hypoxia is an important micro-environmental factor that leads to malignant progression by increasing tumor stemness (19). Several stemness-related genes, whose expressions rely on the orchestration of epigenetic signatures and miRNA/miRNA posttranscriptional networks (2), are required to program either embryonic stem cells or tumor cells into pluripotency under hypoxia (20). Therefore, hypoxia is a suitable testing model to demonstrate the regulation of cancer stemness.

Several unanswered questions remain with regard to inducing stem-like properties in cancer cells. First, the detailed mechanism by which DDX17 promotes stem-like properties is unknown. Second, it is unclear whether DDX17 could be ubiquitinated and whether the ubiquitination site could control stem-like characteristics, which, in turn, affect therapeutic efficacy in cancer treatment. Finally, DDX17 is an important factor in both transcriptional activation and miRNA biogenesis (21); however, the question remains which of these two machineries, transcriptional or post-transcriptional or both, regulates cancer stem-like properties.

Here we present a unifying model that K63-polyubiquitinated DDX17 can control miRNA biogenesis and histone modification to induce the expressions of stemness-related genes. This study uncovers a precise mechanism by which a RB plays a central role in coordinating posttranscriptional and transcriptional machineries to define cancer stem-like features and provides a therapeutic venue for targeting ubiquitination of a RB.

Materials and Methods

Cell culture and oxygen deprivation

Human head and neck cancer FaDu and OECM-1, lung cancer H1299, and breast cancer MCF7 cells were obtained from ATCC and MOR/CPR lung cancer cell line from the European Collection of Cell Cultures. Cell line authentication was done by Food and Industry Research & Development Institute (Taiwan) using short random repeat DNA sequencing. The human embryonic kidney 293T cell line was obtained as described previously (22). Primary human bronchial epithelial cells (NHK-SCC) cells were kindly provided by Dr. M.-H. Yang (National Yang-Ming University, Taipei, Taiwan; ref. 23). Head and neck cancer stem cells (with ALDH1) (National Yang-Ming University, Taipei, Taiwan; ref. number: 2017-042). Head and neck cancer FaDu and OECM-1, lung cancer H1299, and breast cancer MCF7 cells were obtained from ATCC and MOR/CPR lung cancer cell line from the European Collection of Cell Cultures. Cell line authentication was done by Food and Industry Research & Development Institute (Taiwan) using short random repeat DNA sequencing. The human embryonic kidney 293T cell line was obtained as described previously (22). Primary human bronchial epithelial cells (NHK-SCC) cells were kindly provided by Dr. M.-H. Yang (National Yang-Ming University, Taipei, Taiwan; ref. 23). Head and neck cancer FaDu and OECM-1, lung cancer H1299, and breast cancer MCF7 cells were obtained from ATCC and MOR/CPR lung cancer cell line from the European Collection of Cell Cultures. Cell line authentication was done by Food and Industry Research & Development Institute (Taiwan) using short random repeat DNA sequencing. The human embryonic kidney 293T cell line was obtained as described previously (22). Primary human bronchial epithelial cells (NHK-SCC) cells were kindly provided by Dr. M.-H. Yang (National Yang-Ming University, Taipei, Taiwan; ref. number: 2017-042).

Cell culture and oxygen deprivation

The pCM6-Myc-DDX17 plasmid was purchased from Addgene. The pCM6-HA-HIF1α and pCM6-HA-MOR/CPR plasmids were purchased from Celprogen. The cell lines showed negative contamination. Oxygen deprivation was achieved by adding 50 µM of DMEM at the indicated densities and were mixed with an equal volume of Matrigel (Corning). The mixture was subcutaneously injected into the lateral flanks of 6-week-old female Balb/c nude mice. Mice were sacrificed 8 weeks after inoculation of tumor cells. The tumor weights were measured after harvesting the xenotransplantation. All animal studies were approved by the Institutional Animal Care and Use Committee, China Medical University (Taichung, Taiwan; reference number: 2017-042).

Quantitative chromatin immunoprecipitation

Cells were cross-linked with 1% formaldehyde for 10 minutes and stopped by glycerol to a final concentration of 0.125 mol/L (24). Fixed cells were washed twice with Tris buffered saline (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl) and harvested in 5 mL of SDS buffer (50 mmol/L Tris, pH 8.0, 0.5% SDS, 100 mmol/L NaCl, 5 mmol/L EDTA, and protease inhibitors). Cells were pelleted by centrifugation and suspended in 2 mL of IP buffer (100 mmol/L Tris, pH 8.6, 0.3% SDS, 1.7% Triton X-100, 5 mmol/L EDTA). Cells were sonicated with a 0.25-inch diameter probe for 15 seconds twice using a Bioruptor Pico sonicator (Diagenode). For each immunoprecipitation, 1 mL of lysate was preclarified by adding 50 µL of blocked protein A beads (50% protein A-Sepharose, Amersham Biosciences; 0.5 mg/ml BSA, 0.2 mg/ml salmon sperm DNA) at 4°C for 1 hour. Samples were spun, and the supernatants were incubated at 4°C for 3 hours with no antibody, IgG, or antibodies to be tested. Immune complexes were recovered by adding 50 µL of blocked protein A beads and incubated overnight at 4°C. Beads were successively washed with (i) mixed micelle buffer (20 mmol/L Tris, pH 8.1, 150 mmol/L NaCl, 5 mmol/L EDTA, 5% w/v sucrose, 1% Triton X-100, 0.2% SDS), (ii) buffer 500 (50 mmol/L HEPES, pH 7.5, 0.1% w/v deoxycholic acid, 1% Triton X-100, 500 mmol/L NaCl, 1 mmol/L EDTA), (iii) LiCl detergent wash buffer (10 mmol/L Tris, pH 8.0, 0.5% deoxycholic acid, 0.5% Nonidet P-40, 250 mmol/L LiCl, 1 mmol/L EDTA), and (iv) TE buffer (10 mmol/L Tris, 1 mmol/L EDTA)
and then eluted with 1% SDS and 0.1 mol/L NaHCO₃. Twenty microliters of 5 mol/L NaCl was added to the eluates, and the mixture was incubated at 65°C for 4 hours to reverse the cross-linking. After digestion with proteinase K, the solution was phenol/chloroform-extracted and ethanol-precipitated. DNA fragments were resuspended in 50 μL of water and 5 μL was used in a PCR reaction. For quantitative chromatin immunoprecipitation (qChIP) assay, DNA samples were quantified by the SYBR Green assay using SYBR Green PCR Master Mix (Applied Biosystems) with specific primer. Data were analyzed by the C_i method and plotted as % input DNA. qChIP values were calculated by the following formula: % input recovery = [100/(input fold dilution/bound fold dilution)] × 2(input CT – bound CT). The antibodies used are listed in Supplementary Table S3. The primers used in qChIP assay are listed Supplementary Table S5.

**In vitro sphere formation**

Cells (1 × 10⁵) were seeded and incubated with serum-free medium composed of DMEM/F12 (Thermo Fisher Scientific), N2 supplement (Thermo Fisher Scientific), B-27 supplement (Thermo Fisher Scientific), 10 ng/ml human recombinant basic fibroblast growth factor (Thermo Fisher Scientific), and 10 ng/ml EGF (Thermo Fisher Scientific) in a 12-well plate. The spheroids were resuspended to form secondary and tertiary spheroids. Tumor spheres with a size >100 μm were counted. The number of spheroids was counted after 14 days.

**In vitro clonogenic assay**

Briefly, cells (2 × 10³) were seeded in 0.6% agarose supplemented with 10% FBS DMEM. After 3 weeks of incubation, colonies were fixed with 3.7% paraformaldehyde for 30 minutes at room temperature and stained by 0.01% crystal violet.

**Flow cytometry analysis and sorting**

To determine the ALDH1 expression, cells were stained with the ALDEFLUOR Assay Kit (StemCell Technologies). A specific ALDH1 inhibitor, diethylaminobenzaldehyde, was used as a negative control, and cells were analyzed with a FACSCalibur flow cytometer. A total of 1 × 10⁷ cells were stained with the ALDEFLUOR assay kit and subjected to sorting by BD FACSaria III (BD Biosciences).

**Luciferase reporter assays**

The reporter constructs of BMHI-3’UTR, SOX2-3’UTR, and OCT4-3’UTR and their mutated constructs were transfected into H1299/WT and H1299/K190R-stable cells or 293T/sci-si and 293T/HectH9-si–stable cells. pMIR expression vectors were cotransfected with DDX17-WT or DDX17-K190R expression plasmids into 293T cells accordingly. The reporter constructs of BMHI promoter, SOX2 promoter, and OCT4 promoter were cotransfected with DDX17-WT or DDX17-K190R expression plasmids into 293T cells. A pCMV-β-gal plasmid was used as an internal control. The luciferase activities were assayed as described previously (22).

**Lentivirus siRNA and cDNA overexpression experiments**

Lentivirus containing short hairpin RNAs (shRNA) expressed in the lentiviral vector (pLKO.1-puro) or containing designated cDNAs were generated in 293T cells as described previously (25). The sequence and clonal names of plasmid pLKO-scrambled or other plKO plasmids against HectH9 and DDX17 were described in Supplementary Table S6. These LKO plasmids and packaging plasmid pCMVΔR8.91 and pMD.G were provided by National RNAi Core Facility of Academia Sinica (Taipei, Taiwan). For lentivirus production, 293T cells were transfected with 5 μg pLKO.1-puro lentiviral vectors expressing different shRNAs along with 0.5 μg of envelope plasmid pMD.G and 5 μg of packaging plasmid pCMVΔR8.91, whereas for overexpression, the amounts of plasmids were doubled at transfection. Virus was collected 48 hours after transfection. To prepare HectH9 or DDX17 knockdown/overexpression cells, H1299, OECM-1, or FaDu cells were infected with lentivirus for 24 hours, and stable clones were generated by selection with appropriate antibiotics.

**Isolation of K63-ubiquitinated proteins**

K63-ubiquitinated proteins were isolated from cell lysate using Flag-K63-TUBE (K63-Tandem Ubiquitin Binding Entities; Life Sensors, catalog no. UM6040). The experimental process was conducted according to the manufacturer’s protocol. Briefly, to pull down proteins, cells were lysed with standard lysis buffer with additional 100 μmol/L K63-TUBE peptide, 100 μmol/L PR619, 2.5 mmol/L o-PA, and 5 mmol/L NEM. The cell lysate was incubated for 2 hours at 4°C with the TUBE peptide. K63 proteins were immunoprecipitated using Flag-M2 agarose affinity beads at 4°C overnight. Ubiquitinated proteins were eluted with 0.2 mol/L glycine HCl (pH 2.5) for 1 hour at 4°C. The pH of the eluate was neutralized by 1 mol/L Tris (pH 8.0) for proteomic analysis. Ten-fold the eluates were boiled in Laemmli buffer for Western blotting.

**In-gel digestion**

The protein samples from SDS-PAGE were subjected to typical in-gel digestion. The gels were cut into small gel pieces and washed three times with 25 mmol/L TEAB containing 50% (v/v) ACN followed by dehydration with 100% ACN and completely drying by vacuum centrifugation. Then, protein samples were digested by Trypsin (protein:trypsin = 50:1, g/g) in 25 mmol/L TEAB at 37°C overnight. The extraction of tryptic peptides was performed three times with 5% (v/v) FA in 50% (v/v) ACN for 30 minutes and dried completely by vacuum centrifugation at room temperature.

**LC/MS-MS analysis**

Peptides were reconstituted in buffer A (0.1% formic acid) and analyzed by using LTQ-Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) with a nanospray interface. Peptide samples were directly loaded onto a 75 μm × 250 mm Thermo Scientific Acclaim PepMap100 C18 Nano LC Column. The mass spectrometer was operated in the data-dependent mode with a Top speed method. Tandem MS was performed with an isolation window of 1.6 kDa using quadrupole and CID fragmentation with collision energy of 30. The second biological replicates were analyzed using the TripleTOF 5600 system (AB SCIEX) with a nanoACQUITY UPLC (Waters) and were applied with 15 cm × 100 μm self-pulled column packed with C18-AQ particles (Dr. Maisch). Peptides were separated through a gradient of up to 80% (vol/vol) buffer B (acetonitrile with 0.1% formic acid) over 120 minutes at the flow rate of 500 nL/minute. Data were acquired using data-dependent mode, top 10 precursor ions were selected on the basis of exceeding a threshold of 100 cps in each MS survey scan, and 10 MS/MS scans were performed for 200 ms each. The
collision energy was adjusted automatically by the rolling CID function of Analyst TF 1.5 and dynamic exclusion was set at 6 seconds.

Protein identification and quantitation by MaxQuant
The raw files were processed with MaxQuant (version 1.5.1.11) and MS/MS spectra searched by Andromeda search engine against the SwissProt human database (April 2014) with the following parameters were allowed: tryptic peptides with 0–2 missed cleavage sites; the parent ion tolerance was 10 ppm and the fragment ion mass tolerance was 0.6 kDa; oxidation (M) was set as variable modifications. Search results were processed with MaxQuant and filtered with a false discovery rate of 0.01. Label-free quantitation (LFQ) were obtained through MaxQuant. The match between run option and the LFQ quantitation were activated.

Coimmunoprecipitation assays and GST pull-down assays
Coimmunoprecipitation experiments were performed by incubating different antibodies (Supplementary Table S3) overnight with 0.5–1 mg of whole-cell lysates prepared by lysis in 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L phenylmethylsulfonyl-fluoride, 25 mmol/L NaF, and protease inhibitors, from H1299, MCF7, and 293T cells overexpressing proteins of interest. The immune complexes were incubated with protein-A/G beads at 4°C for 2 hours and preblocked with 10% BSA. The immunoprecipitates were washed three times with the same lysis buffer, mixed with 1 × Laemmli dye, boiled for 5 minutes, and loaded on SDS-polyacrylamide gels (SDS-PAGE). After transfer, the filters were blocked with blocking buffer, probed with primary and secondary antibody sequentially, and developed. The GST pull-down assay was performed by incubating Flag-DDX17-WT, K63-Ub-Flag-DDX17-WT, or Flag-DDX17-K190R protein with GST-p300-CH3-WT or GST-p300-CH3-AZZ protein (or GST protein as a control) and Glutathione-Agarose (Sigma). K63-Ub-Flag-DDX17-WT protein was obtained by cotransfecting Flag-DDX17-WT with HA-ubiquitin-K63 plasmids into 293T cells. All the Flag constructs were purified by anti-Flag M2 agarose and eluted with 3 × Flag peptides. Purification of GST proteins was performed as described previously (24). The pulled down Flag-DDX17 protein was detected by Western blot analysis.

Identification of miRNAs targeting the stemness-related genes
The validated and predicted miRNA–gene relationships in this study were based on the pipeline constructed in our previous study (26). In brief, data of experimentally validated miRNA–gene interactions were extracted and identified from miRTARBase (27), a curated database of miRNA–target interactions. For predicted miRNA–gene interactions, 12 bioinformatics tools were used to identify putative miRNAs targeting stemness-related genes. The miRNAs predicted to target stemness-related genes (or anti-stemness miRNAs) by at least 3 algorithms were selected for further experimental validation. As shown in Fig. 4A and Supplementary S4A, 11 miRNAs are identified to target the 6 stemness-related genes.

H3K56Ac ChIP-seq data analysis
The H3K56Ac ChIP-seq dataset was obtained from Roadmap Epigenomics Project at Gene Expression Omnibus (GSE16256). Raw sequencing reads were mapped to the human genome (assembly hg19) using Bowtie v0.12.7 algorithm with m1 and v3 parameters (28). The binding regions of H3K56Ac were identified by MACS v1.4.2 algorithm (29) with default parameter and then annotated by the Bioconductor package, ChIPpeakAnno (30).

Survival analysis
Published microarray data of series from Gene Expression Omnibus (GSE10300, GSE2837, GSE42127, GSE14814; ref. 31) were used to obtain probe-level expression values for HectH9 and the six stemness-related genes in HNSCC (n = 84) and lung adenocarcinoma (n = 204). We used Jetset (32) to select the optimal microarray probe set to represent a gene for those with multiple probes and used variance normalized values (z-score) to indicate the gene expression levels. For survival analysis, each HNSCC or lung cancer was classified as positive of the HectH9-stemness signature if HectH9 and the sum of the z-scores for the expression values of 6 stemness genes were greater than the median of the population. The Kaplan–Meier estimate was used for survival analysis, and statistical significance for differences between survival curves of patients was determined using the log-rank test by the survival package (33) of R (version 3.4.0; ref. 34).

Statistical analyses
Unless otherwise noted, each sample was assayed in triplicate. For in vitro analyses, each experiment was repeated at least three times. All error bars represent SEM and the exact n is stated in the corresponding figure legend. Student t test was used to compare two groups of independent samples. The level of statistical significance was set at 0.05 for all tests.

Data availability
All of the mass spectrometry–derived proteomics datasets have been submitted in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the Jpost partner repository with the dataset identifier JPS1000240 and PXD006059. The data that support the findings of this study are available from the corresponding author upon request.

Results
HIF1α–induced HectH9 confers cancer stemness-like properties in tumor cells
Because hypoxia-induced pluripotency is a good model to explore the underlying mechanisms that contribute to cancer stemness-like features (20), we decided to utilize this experimental system. Because of the overlapping role of hypoxia and HectH9 in regulating cancer stemness (16, 20), we tested whether HectH9 expression is induced by hypoxia. We found that hypoxic treatment in several tumor cell lines (including head and neck: FaDu and OECM-1; lung: H1299; and breast: MCF7) and overexpression of a constitutively-active HIF1α(R2DO) construct in 293T cells led to an upregulation of HectH9 mRNA and protein levels (Fig. 1A; Supplementary Fig. S1A). Promoter characterizations showed that hypoxia-response element (HRE) mutation at −340−−336 of HectH9 promoter abolished activation of HectH9 expression in a reporter gene assay, suggesting that HIF1α could transcriptionally regulate HectH9 expression (Supplementary Fig. S1B). To investigate whether HectH9 plays a role in mediating tumor stemness, we knocked down HectH9 expression in OECM-1 and H1299 cells and conducted the tumor sphere assays (Fig. 1B; Supplementary Fig. S1C). Suppression of HectH9
HectH9 knockdown reduced ALDH1 expression in primary culture of head and neck squamous cell carcinoma (HNSCC) cells and primary head and neck cancer stem cells (CSC) as HectH9 knockdown reduced the sphere numbers of these tumor cells (Supplementary Fig. S1M).

We further examined the role of HectH9 in regulating HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness.

Figure 1. Hypoxia/HIF1α–upregulated HectH9 plays a role in tumor stem-like properties. A, qRT-PCR analysis (left) for endogenous HectH9 mRNA levels and Western blot analysis (right) for HectH9 protein levels under normoxia (N) or hypoxia (H) in FaDu, OECM-1, H1299, and MCF7 cells. VEGF served as the positive control for hypoxia for qRT-PCR. Data are represented as the mean ± SEM for biological triplicate experiments. B, Western blot analysis to test HectH9 knockdown in OECM-1 cells with either control or two HectH9 shRNAs. C, Left, representative pictures of tumor sphere formation of control and HectH9-knockdown clones of OECM-1 cells. Right, representative pictures of colony formation of control and HectH9-knockdown clones of OECM-1 cells. Scale bar, 100 μm. D, Left, number of tumor sphere formation activity of control versus HectH9 knockdown clones of OECM-1 cells measured by primary, secondary, and tertiary spheres. Right, number of colony formation of control versus HectH9 knockdown clones of OECM-1 cells. Data are represented as the mean ± SEM for biological triplicate experiments. E, The population of ALDH1+ cells analyzed by flow cytometry in control versus HectH9 knockdown clones of OECM-1 cells. F, Western blot analysis to test HIF1α(AODD) overexpression and HectH9 knockdown in FaDu cells. G, Representative pictures of tumor sphere formation of the indicated stable clones of FaDu cells. H, Number of tumor sphere formation activity of the indicated stable clones of FaDu cells measured by primary, secondary, and tertiary spheres. Scale bar, 100 μm. Data are represented as the mean ± SEM for biological triplicate experiments. I, Representative pictures of subcutaneous implantation at 10^3 of the indicated stable clones in FaDu cells. n = 6/group. Scale bar, 10 mm. J, Average tumor weight of the in vivo tumor-initiating assay at 10^3 cell implantation (n = 6 mice/group). The control weight was arbitrarily set as 1, *P < 0.05, statistical significance between experimental and control clones, t test. #, there was no tumor formation.

significantly decreased the number of spheres and colonies (Fig. 1C and D; Supplementary Fig. S1D and S1E). HectH9 knockdown mildly decreased proliferation without a change in apoptosis (Supplementary Fig. S1F and S1G), suggesting that proliferation and apoptosis may not be the main causes for reduced colony numbers. Flow cytometry analyses revealed that HectH9 knockdown reduced ALDH1+ population (Fig. 1E; Supplementary Fig. S1H), which represent the stem-like population (35). Next, to evaluate whether HectH9 plays a role in inducing stem-like subpopulation of cancer cells, we suppressed HectH9 expression in ALDH1+ and ALDH1+ cells. Sphere formation in ALDH1+ cells was impaired by HectH9 knockdown compared with controls. (Supplementary Fig. S11 and S1I). The impact of HectH9 on stem-like traits was also validated in the primary culture of head and neck squamous cell carcinoma (HNSCC) cells and primary head and neck cancer stem cells (CSC) as HectH9 knockdown reduced the sphere numbers of these tumor cells (Supplementary Fig. S1K–S1M).

We further examined the role of HectH9 in regulating HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness. We knocked down HectH9 in HIF1α–induced tumor stemness.

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was knocked down in HIF1α (Fig. 1A). Hypoxia can induce HectH9, whose expression regulates tumor stem-like property and tumor-stemness requiring the enzymatic activity and an effector substrate. To characterize possible substrates of HectH9, we immunoprecipitated K63-linked ubiquitinated proteins with Flag-tagged K63-specific tandem ubiquitin-binding entity (K63-UIM; ref. 36) in H1299/HectH9-si and H1299/scr-si cells and identified them by LC/MS-MS (Fig. 2A; Supplementary Fig. S2A). Among the 29 proteins identified in replicates, 72% of the putative substrates belong to the RNA metabolic process pathway by Gene Ontology (http://www.geneontology.org/; Fig. 2B; Supplementary Table S8). We chose DDX17 for further characterization because DDX17 is an RNA helicase and plays a significant role in RNA metabolism that may regulate miRNA processing and transcription (5, 9, 21). Supporting the rationale to test tumor stem-like property regulated by DDX17, we found that suppression of DDX17 attenuated the number of sphere formation in (ΔODD)-expressing clones and found that while HIF1α (ΔODD) increased the sphere numbers, knockdown of HectH9 significantly decreased the sphere numbers (Fig. 1F–H). The tumor-initiating ability was compromised in vivo when HectH9 was knocked down in HIF1α(ΔODD)-expressing FaDu cells at 10³ cell inoculation (Fig. 1I and J; Supplementary Table S7). Together, these data indicate that HIF1α could induce HectH9, whose expression regulates tumor stem-like property and tumor-initiating capability.

K63-linked polyubiquitination of DDX17 mediated by HectH9

Because HectH9 is an E3 ligase involved in K63-linked polyubiquitination (15, 24), we hypothesized that HectH9-mediated tumor stemness requires the enzymatic activity and an effectorsubstrate. To characterize possible substrates of HectH9, we immunoprecipitated K63-linked ubiquitinated proteins with Flag-tagged K63-specific tandem ubiquitin-binding entity (K63-UIM; ref. 36) in H1299/HectH9-si and H1299/scr-si cells and identified them by LC/MS-MS (Fig. 2A; Supplementary Fig. S2A). Among the 29 proteins identified in replicates, 72% of the putative substrates belong to the RNA metabolic process pathway by Gene Ontology (http://www.geneontology.org/; Fig. 2B; Supplementary Table S8). We chose DDX17 for further characterization because DDX17 is an RNA helicase and plays a significant role in RNA metabolism that may regulate miRNA processing and transcription (5, 9, 21). Supporting the rationale to test tumor stem-like property regulated by DDX17, we found that suppression of DDX17 attenuated the number of sphere formation in...
both H1299 and OECM-1 cells (Supplementary Fig. S2B–S2D). In accordance with HectH9 suppression, DDX17 knockdown slightly reduced proliferation but did not change apoptosis (Supplementary Fig. S2E and S2F). To test whether DDX17 is a bona fide substrate of HectH9, we overexpressed HectH9-wild type or catalytically inactive HectH9 (HectH9-CS; ref. 37) and found increased DDX17 polyubiquitination with the wild-type HectH9, but not with HectH9-CS (Supplementary Fig. S2G). Coimmunoprecipitation experiments revealed that HectH9 and DDX17 could endogenously interact with each other in vivo (Fig. 2C). To corroborate the ubiquitination status of DDX17 by HectH9 under hypoxia, we immunoprecipitated DDX17 and found that hypoxia induced K63-linked polyubiquitination of DDX17, which was decreased when HectH9 was suppressed, whereas K48-linked polyubiquitination moieties on DDX17 were not perturbed (Fig. 2D), suggesting that hypoxia-mediated HectH9 does not control DDX17 protein degradation. Indeed, the cycloheximide chase assay showed that DDX17 was a stable protein under HectH9 suppression (Supplementary Fig. S2H). Collectively, these data demonstrate that DDX17 is K63-linked polyubiquitinated by HectH9 under hypoxia.

Next, to identify the polyubiquitination site on DDX17 mediated by HectH9, we truncated DDX17 into DDX17-ΔC and DDX17-ΔN and coexpressed them with V5-HectH9 and HA-Ub plasmids in 293T cells. We found that the polyubiquitination moieties were mainly located at the N-terminal fragment of DDX17 encompassing the helicase domain under HectH9 overexpression (Supplementary Fig. S2I). Further truncation of the N-terminal fragment indicated that ubiquitination of fragment 101–200 was elevated by either HectH9 overexpression (Supplementary Fig. S2J) or hypoxia (Supplementary Fig. S2K). Individual lysine residue mutation showed that only Flag-DDX17-101–200K190R abolished the HectH9-mediated polyubiquitination signal (Supplementary Fig. S2L). To validate the polyubiquitination status under hypoxia, we coexpressed full-length DDX17 WT or DDX17-K190R with two mutants of ubiquitin (K48R or K63R) followed by hypoxia exposure. We found that only K48R-ubiquitin was ligated to DDX17-WT but not to DDX17-K190R under hypoxia (Fig. 2E, left). On the contrary, the K63R-ubiquitin mutant could not form the ubiquitin chains on either DDX17-WT or DDX17-K190R (Fig. 2E, right).

We next generated DDX17-WT and DDX17-K190R-expressing stable clones by lentivirus in H1299 and FaDu cells (Supplementary Fig. S2M). Cells with K190R showed significantly reduced sphere and colony formation abilities compared with WT (Fig. 2F and G), with a slight decrease in proliferation and no difference in apoptosis (Supplementary Fig. S2N and S2O). Therefore, these data suggest that DDX17 polyubiquitination at K190 may play an important role in HectH9-mediated tumor stem-like properties.

**Nonubiquitinated DDX17-K190R reduces cancer stemness properties and tumor-initiating capabilities**

To further characterize the downstream effectors in stemness, we screened a group of stemness-related genes associated with hypoxia (20, 38) in HectH9- and DDX17-knockdown cells. *OCT4*, *SOX2*, *NANOG*, *BMI1*, *NOTCH1*, and *NOTCH2* were concurrently downregulated but not WNT1 (Fig. 3A; Supplementary Fig. S3A), suggesting that *OCT4*, *SOX2*, *NANOG*, *BMI1*, *NOTCH1*, and *NOTCH2* may be the downstream targets of HectH9 and DDX17. We next confirmed that these six stemness-related genes were upregulated at the RNA and protein levels under hypoxia. (Fig. 3B; Supplementary Fig. S3B and S3C). Knockdown of each of *OCT4*, *SOX2*, *NANOG*, *BMI1*, *NOTCH1*, and *NOTCH2* gene reduced the sphere formation (Supplementary Fig. S3D), indicating that these six genes are important in driving cancer stemness in our cell line model.

Next, we examined the effect of ubiquitination of DDX17 on these stemness-related genes in WT or K190R-stable clones in H1299 and FaDu cells. The RNA levels of *OCT4*, *SOX2*, *NANOG*, *BMI1*, *NOTCH1*, and *NOTCH2* were decreased in K190R compared with WT, respectively (Fig. 3C and D). The protein levels of these stemness-related genes in K190R cells were also reduced in accordance with their RNA levels (Fig. 3E and F). Flow cytometry revealed that K190R clones possessed less ALDH1α population than WT cells (Fig. 3G and H). The in vivo tumor initiation study showed that FaDu/K190R reduced tumor development at low-dose (10³) implantations compared with the wild-type counterpart (Fig. 3I and J; Supplementary Table S7). Because the stem-like feature is associated with drug resistance in cancer (39), we investigated whether HectH9 or DDX17 ubiquitination would affect drug sensitivity in cisplatin-resistant MOR/CPR cells. The data showed that HectH9 knockdown or K190R overexpression in MOR/CPR cells increased cisplatin sensitivity by inducing apoptosis (Fig. 3K and L; Supplementary Fig. S3E–S3I), implicating that the HectH9–DDX17 axis might be the therapeutic targets in cancer treatment.

**DDX17 ubiquitination regulates miRNAs biogenesis targeting stemness-related genes**

Because DDX17 plays a role in miRNA biogenesis (9), which is involved in tumor stemness (40), we examined whether HectH9-mediated polyubiquitination of DDX17 can alter the activity of miRNA biogenesis. We utilized bioinformatics approaches to identify the experimentally validated and predicted miRNA–gene interactions of the six stemness-related genes (the detailed information is described in Materials and Methods, Fig. 4A, and Supplementary Fig. S4A). The activity of the Microprocessor can be determined by comparing the amount of pri-miRNAs with that of mature miRNAs (9). Quantification by qRT-PCR showed that representative mature miRNAs were repressed under hypoxia (Fig. 4B, left; Supplementary Fig. S4B and S4C, left), with corresponding sustained or accumulated pri-miRNA expression (Fig. 4B, right; Supplementary Fig. S4B and S4C, right), indicating that hypoxia reduces the activity of the Microprocessor. To examine whether the hypoxia-reduced activity of the Microprocessor is mediated by the HectH9–DDX17 pathway, we next characterized the miRNA status in HectH9-expressing cells. Similar to hypoxia-treated cells, HectH9 overexpression consistently reduced mature miRNA levels with sustained or accrued pri-miRNA levels (Fig. 4C). To demonstrate that these miRNAs play a role in HectH9-mediated tumor stem-like characteristics, we replenished miR-16 or miR-34a in HectH9-overexpressing cells as an example and found that overexpression of miR-16 or miR-34a decreased the sphere and colony numbers in HectH9-overexpressing cells (Supplementary Fig. S4D–S4G). On the contrary, suppression of HectH9 increased mature miRNA levels without concordant changes at the pri-miRNA levels (Supplementary Fig. S5A) and further knockdown of miR-16 or miR-34a increased the sphere and colony numbers in HectH9-knockdown cells (Supplementary Fig. S5B–S5E). These data suggest that HectH9 can modulate hypoxia-induced tumor stem-like traits via miRNA biogenesis.
Next, to characterize whether DDX17 ubiquitination regulates miRNA biogenesis, we examined the expression levels of the representative miRNAs in DDX17-WT and DDX17-K190R cells. We found that nonubiquitinated DDX17 mutants significantly induced more mature miRNA productions compared with the wild-type, without concordant changes at the pri-miRNA levels (Fig. 4D). Antagonizing miR-16 or miR-34a expression resulted in wild-type, without concordant changes at the pri-miRNA levels induced more mature miRNA productions compared with the knockout of HectH9 in DDX17-WT further suppressed the overexpression clones in FaDu cells (n = 6 mice/group). The control weight was arbitrarily set as 1. *P < 0.05 between DDX17 WT and DDX17-K190R (t test). J, Tumor-killing effect of cisplatin using the MTS assay. Data are represented as the mean ± SEM for biological triplicate experiments. *P < 0.05 (t test). K, The apoptosis ratio of the stable clones in H1299/WT and H1299/K190R clones. G and H, The population of ALDH1+ cells analyzed by flow cytometry in WT and K190R clones in H1299 (G) and FaDu (H) cells. I, Representative pictures of subcutaneous implantation at 10^5 of FaDu/WT and FaDu/K190R cells. J, Western blot analysis to detect the protein levels of stemness-related genes under normoxia (N) or hypoxia (H) in H1299 cells. Data are represented as the mean ± SEM for biological triplicate experiments. *P < 0.05 between DDX17-WT and DDX17-K190R (t test). K, Western blot analysis of stemness-related gene expressions in H1299/WT and H1299/K190R clones. L, Western blot analysis of stemness-related gene expressions in HectH9- (top) or DDX17-knockdown (bottom) H1299 cells. Data are represented as the mean ± SEM for biological triplicate experiments. *P < 0.05 between DDX17-WT and DDX17-K190R (t test).

To demonstrate the posttranscriptional effect of DDX17 ubiquitination, we examined the luciferase activities harboring the 3'UTR of three representative genes, BMI1, SOX2, and OCT4, in DDX17-WT and K190R cells. The activities of BMI1, SOX2, and OCT4-3'UTR were repressed in K190R cells at a significant level compared with DDX17-WT (Fig. 4E). In line with this finding, knockdown of HectH9 in DDX17-WT further suppressed the luciferase activities of BMI1, SOX2, and OCT4-3'UTRs compared with DDX17-WT alone (Supplementary Fig. S5I), implicating that...
ubiquitination of DDX17 may affect BMI1, SOX2, and OCT4 expressions via miRNA biogenesis. To corroborate this notion, mutations affecting miRNA binding sites in representative stemness genes, including miR-34a and miR-128 for BMI1-3'UTR (41, 42) and miR-203b (43) for OCT4-3'UTR, elevated the luciferase activities of BMI1- and OCT4-3'UTR reporter constructs in both DDX17-WT and DDX17-K190R cells (Supplementary Fig. S5K), demonstrating that stemness genes containing the mutated miRNA binding sites could abolish the effects of DDX17-processed miRNAs on these genes.

Together, these data demonstrate that DDX17 ubiquitination could regulate miRNA biogenesis and processing. Because DDX17 expression does not change under hypoxia, we infer that hypoxia-induced DDX17 ubiquitination may affect the interaction of DDX17 with the Microprocessor.

Ubiquitinated DDX17 associates with a ubiquitin-binding protein, p300, and YAP and dissociates from the Drosha–DGCR8 complex. Sequestration of DDX17 from Microprocessor by YAP in the Hippo pathway in a cell density-dependent manner and increased nuclear presence of YAP under hypoxia have been reported (9, 44). To observe the localization of YAP under hypoxia, we conducted nuclear/cytosol fractionation and immunoblotting with anti-phospho-S127 antibodies (44). Hypoxia induced YAP translocation into the nucleus via decreasing the

Figure 4.
Nonubiquitinated DDX17 promotes the miRNA biogenesis of a subset of anti-stemness miRNAs. A, The miRNA–gene network showing miRNAs targeting to the stemness-related genes based on experimentally validated evidence (solid line) and predicted algorithms (dashed line). The thickness of the lines indicates the power of association, which is based on the number of evidence or algorithms. B, Left, qRT-PCR analysis of mature miRNA levels normalized to RNU-48 in H1299 cells after cells were treated with normoxia or hypoxia. Right, relative pri-miRNA expression measured by qRT-PCR analysis of mature miRNA levels normalized to RNU-48 in HectH9-overexpressing H1299 cells. C, Left, qRT-PCR analysis of mature miRNA levels normalized to RNU-48 in H1299 cells. Right, relative pri-miRNA expression measured by qRT-PCR normalized to RNU-48 in HectH9 in the same cells. Inset, HectH9 expression as the positive control of the experiment. Data are represented as the mean ± SEM for biological triplicate experiments. D, Left, qRT-PCR analysis of mature miRNA levels normalized to RNU-48 of DDX17-WT or DDX17-K190R–expressing H1299 cells. Right, relative pri-miRNA expression measured by qRT-PCR normalized to RNU-48 in HectH9 in the same cells. Data are represented as the mean ± SEM for biological triplicate experiments. E, Luciferase assays with a BMI1-, SOX2-, or OCT4–3'UTR reporter in DDX17-WT- and DDX17-K190R–expressing cells. Luciferase activity was normalized to that of pCMV-b-gal. Data are represented as the mean ± SEM for biological triplicate experiments. *P < 0.05, statistical significance between experimental and control clones (t test).
levels of phosphorylated YAP (Fig. 5A; Supplementary Fig. S6A and S6B). These results indicate that nuclear YAP may participate in sequestration of DDX17 under hypoxia. Because YAP does not contain an acknowledged ubiquitin binding domain (UBD), we searched for other proteins that might also interact with polyubiquitinated DDX17. We analyzed the interactomes of YAP and DDX17 using the BioGRID database (45) to identify potential ubiquitin-binding protein. Among the eight overlapping proteins, we further characterized p300, which contains a ZZ-type zinc finger (ZZ) domain as a possible UBD (Fig. 5B; ref. 46). Multiple sequence alignments showed that the ZZ domain is conserved among different species (Supplementary Fig. S6C). The coimmunoprecipitation assay showed that under hypoxia, DDX17 increased the association with YAP and p300, but decreased interaction with Drosha and DGC8 (Fig. 5C; Supplementary Fig. S6D). Enhanced interaction under hypoxia between endogenous p300, YAP, and DDX17 was also detected using anti-YAP or anti-p300 antibodies, respectively (Supplementary Fig. S6E and S6F). Overexpression of myc-DDX17 and Flag-p300 followed by immunoprecipitation by anti-myc antibodies

Figure 5.
Increased association of ubiquitinated DDX17 with p300 (the ubiquitin-binding protein), and YAP and reduced association with the Microprocessor. A, Top, Western blot analyses of YAP expression after nuclear and cytosolic fractionation in FaDu cells. GAPDH and H3 served as the cytosol and nucleus control, respectively. Bottom, Western blot analysis of protein of interests under normoxia (N) and hypoxia (H) in FaDu cells. B, Interactome analysis of YAP and DDX17 using BioGRID. C, Detection of the interaction between endogenous YAP, p300, Drosha, DGC8, and DDX17 by coimmunoprecipitation using normoxic (N) or hypoxic (H) FaDu lysates with anti-DDX17 antibodies. IgG was used as a negative control. D, Top, schematic diagram of the domains of the p300 protein. Bottom, detection of the interaction between endogenous DDX17 and truncated p300-CH3 WT or p300-NCBD by immunoprecipitation of DDX17 by anti-DDX17 antibodies. IgG was used as a negative control. E, Top, schematic diagram of the constructs. Bottom, detection of the interaction between endogenous DDX17 and truncated p300-CH3 WT or p300-CH3 ΔZZ, p300-CH3 ΔZZ-TAB2 under normoxia (N) or hypoxia (H) by immunoprecipitation of DDX17 with anti-DDX17 antibodies. F, Detection of the interaction between Flag-p300 and myc-tagged DDX17-WT, Ub-DDX17-WT, or DeltaDDX17 by coimmunoprecipitation with anti-myc antibodies in 293T cells, followed by immunoblotting with the indicated antibodies. For enhancement of K63-polyubiquitinated DDX17 (Ub-DDX17), we further characterized p300, which contains a ZZ-type zinc finger (ZZ) domain as a possible UBD (Fig. 5B; ref. 46).
revealed that DDX17 and p300 could interact with each other and a stronger association was observed under hypoxia (Supplementary Fig. S6C). To examine whether the ZZ-type zinc finger motif (ZZ) of p300 is a ubiquitin-binding motif for the K63 polyubiquitin chains, we coexpressed the CH3 domain of p300 containing the ZZ-type zinc finger motif (a.a. 1643–1857, p300-CH3) or the nuclear coactivator binding domain (a.a. 1858–2153, p300-NCBD) with DDX17 (Fig. 5D, top). The coimmunoprecipitation result showed that only the ZZ motif interacted with DDX17 (Supplementary Fig. S6H) and this interaction was enhanced under hypoxia (Fig. 5D).

To further investigate whether the ZZ motif of p300 is a UBD, we made two constructs, in which the ZZ motif was deleted (ΔZZ), and another where the ZZ motif was replaced by the zinc-finger domain of TAB2 (ΔZZ-TAB2; Supplementary Fig. S6c, ref. 46). The wild-type CH3 domain and the TAB2-replaced CH3 domain mutant (ΔZZ-TAB2) had an increased interaction with DDX17 under hypoxia, but not the ZZ-domain-deleted mutant (ΔZZ; Fig. 5E). Taken together, we found that p300 is a novel ubiquitin-binding protein that interacts with K63-polyubiquitinated DDX17 to enhance the complex formation of DDX17, YAP, and p300 under hypoxia.

Furthermore, to assess whether ubiquitination of DDX17 would affect its interaction with p300, we coexpressed DDX17 WT, polyubiquitinated DDX17 (Ub-DDX17-WT), or DDX17-K190R with p300 and we found that Ub-DDX17-WT enhanced interaction with p300, whereas DDX17-K190R reduced it, as compared with DDX17 WT (Fig. 5F). The GST pull-down assay showed that Flag-DDX17 WT with K63-polyubiquitinated chain interacted with GST-p300-CH3 WT, but not GST-p300-CH3-ΔZZ proteins (lane 5 and lane 8, Supplementary Fig. S6i), demonstrating a direct ZZ domain contact with DDX17 specifically through the K190-conjugated K63-polyubiquitinated chain. Finally, the coimmunoprecipitation assay showed that under hypoxia, DDX17 WT had an increased association with YAP and p300 and a weakened binding with Drosophila and DGC8 (Fig. 5G). On the contrary, DDX17 K190R was retained at the Microprocessor under hypoxia. Overall, these data suggest that under hypoxia, ubiquitinated DDX17 enhanced the complex formation of DDX17, YAP, and p300, and decreased its interaction with the Microprocessor, which may lead to decreased biogenesis of anti-stemness miRNAs.

p300 and DDX17 increase H3K56Ac occupancy on the promoters of stemness-related genes under hypoxia

p300 is a histone acetyltransferase, and hypoxia-induced association between the ubiquitin receptor, p300, DDX17, and YAP leads to the possibility that the complex could together mediate epigenetic marks of histones and hence regulate the stem-like property in tumors. To search for possible correlation, we screened the histone marks in HctH9-knockdown and DDX17-knockdown cells and found that only H3K56 acetylation (H3K56Ac) was concurrently decreased (Supplementary Fig. S7A). This result is in line with the previous evidence that H3K56Ac is implicated in the transcriptional network of pluripotency (47). To look for H3K56Ac-binding regions, we downloaded and reanalyzed the H3K56Ac ChIP-seq data from the Roadmap Epigenomics Project (www.roaddmapepigenomics.org; ref. 48; the detailed information is described in Materials and Methods). We found that H3K56Ac in human embryonic stem cell (hESC) H1 lines was deposited at the regions around transcription start site (TSS) of OCT4, SOX2, NANOG, NOTCH1, and BMI1 (Fig. 6A), implicating that H3K56Ac participates in the regulation of these stemness-related genes.

To verify whether HectH9-mediated ubiquitination of DDX17 modulates the level of H3K56Ac, we knocked down HectH9 in DDX17-overexpressing clones and found that overexpression of DDX17 increased the H3K56Ac levels, whereas further suppression of HectH9 reduced their levels (Fig. 6B). Accordingly, up-regulation of H3K56Ac was detected when p300 was expressed and further enhanced with the presence of DDX17 WT, but the enhanced level of H3K56Ac was abrogated by K190R mutant (Fig. 6C). We next determined whether DDX17/p300/YAP and H3K56Ac will cooccupy at the regions around the TSS of the stemness-related genes. Our qChIP data indicated that under hypoxia, the bindings of DDX17, p300, YAP, and H3K56Ac were elevated at the promoters of BMI1 (at P2), OCT4 (at P3), and SOX2 (at P4; Fig. 6D; Supplementary Fig. S7B). These data suggest that hypoxia-induced ubiquitination of DDX17 may promote the localization of p300 onto their target promoters to incur H3K56Ac. On the contrary, HectH9 suppression resulted in a decrease in the bindings of DDX17, p300, YAP, and H3K56Ac at the promoters of BMI1 (at P2), OCT4 (at P3), and SOX2 (at P4; Supplementary Fig. S7C). Accordingly, the qChIP analysis with anti-Flag antibodies in Flag-DDX17 WT or Flag-DDX17 K190R cells showed that DDX17 WT had a significantly better binding affinity than DDX17 K190R on these promoters (P2 of BMI1, P3 of OCT4, and P4 of SOX2; Fig. 6E). Although the H3K56Ac level was perturbed by HectH9 or DDX17 suppression (Supplementary Fig. S7A), the nonpromoter regions of BMI1, SOX2, and OCT4 rich with H3K56Ac were not affected by HectH9 suppression (Supplementary Fig. S7D), implicating that H3K56Ac may mainly modulate the promoter regions of its target genes.

Because our previously identified deubiquitinase, HAUSP, also a substrate of HectH9, mediates the H3K56Ac levels on the EMT genes, for example, TWIST1, and associates with the super elongation complex (24), we examined whether DDX17 plays a role in TWIST1 regulation. The qChIP data shows that under hypoxia, the binding of DDX17 on the TWIST1 promoter did not change (Supplementary Fig. S7E). Coimmunoprecipitation analyses show that DDX17 did not interact with HAUSP and HIF1α, nor did DDX17 show altered binding with the components of the super elongation complex (AFT4, RNA PolII; Supplementary Fig. S7F and S7G). Moreover, HAUSP occupancy was not changed on the promoter regions of BMI1, OCT4, and SOX2 by hypoxia (Supplementary Fig. S7B). These data suggest that DDX17 and HAUSP may form different complexes to mediate their own individual downstream genes.

Finally, to demonstrate a direct transcriptional effect of DDX17 polyubiquitination on targeted genes, we cloned the parts of OCT4, SOX2, and BMI1 promoters encompassing DDX17-binding regions and checked their luciferase activities. Our data show that DDX17 WT had elevated luciferase activities, whereas DDX17 K190R did not (Supplementary Fig. S7H), suggesting that non-ubiquitinated DDX17 K190R possesses an attenuated transcriptional coactivation ability, which might be due to K190R’s reduced binding to p300 (Fig. 5E) and the reduced level of H3K56Ac. Accordingly, the nascent RNA levels of the stemness-related genes were downregulated by HectH9 suppression (Supplementary Fig. S7I). Together, these results suggest that ubiquitinated...
DDX17/p300/YAP elevates H3K56Ac levels on the promoters of stemness-related genes under hypoxia and transcriptionally regulates the expression of stemness-related genes.

Coexpression of HectH9 and six stemness-related genes is a prognostic factor for patients with HNSCC or lung adenocarcinoma

Finally, we tested whether the HectH9-DDX17-six stemness-related genes pathway provides prognostic implications at the clinical level. Because DDX17 protein stability was not subject to HectH9-mediated ubiquitination, we analyzed the survival difference of patient groups bearing HectH9 and/or stemness marker expression. HectH9 alone or six stemness-related genes alone did not predict the clinical survival outcome in patients with either HNSCC or lung adenocarcinoma (Supplementary Fig. S7I and S7K), suggesting that either HectH9 or six stemness-related genes alone may not be a useful prognostic factor in these two tumor types. However, when the HectH9 and six stemness-related gene...
signature was highly coexpressed in the patients having these two tumor types, the doubled high expression served as a significant prognostic marker to predict shorter disease-free or overall survival than those whose cancers did not coexpress HectH9 and the six stemness markers (Fig. 6F; Supplementary Fig. S7L). Collectively, these data suggest that coexpression of HectH9 and six stemness-related genes could serve as a prognostic marker in predicting the survival of patients with HNSCC or lung adenocarcinoma.

In sum, we report that hypoxia-mediated ubiquitination of DDX17 concerts machineries at the posttranscriptional and transcriptional levels, thereby controlling tumor stem-like property and tumor-initiating capability (a model is presented; Fig. 6G).

### Discussion

Our findings provide a detailed understanding of the mechanisms by which hypoxia-induced PTM serves as a central mechanism to control cancer stem-like features via miRNA biogenesis downregulation and histone modification. To our best knowledge, this report is the first to demonstrate that a single PTM can negatively and positively modulate two downstream machineries, where both events synergistically increase cancer stem-like properties.

We demonstrate that K63-polyubiquitinated DDX17 interacts with different protein partners to dictate either miRNA biogenesis or histone modification mechanisms. The fate of association and dissociation of polyubiquitinated DDX17 is determined, in part, by the presence of nuclear and cytoplasmic YAP. As hypoxia decreases phosphorylated YAP through the SIAH2–LATS signaling pathway (44), nuclear presence of YAP increases (Fig. 5A; Supplementary Fig. S6A and S6B). Nuclear YAP likely sequesters DDX17, causing DDX17 to dissociate from the Drosha–DGCR8 complex, thereby inhibiting miRNA biogenesis. Furthermore, polyubiquitination of DDX17 enhances interaction with p300, a ubiquitin binding protein, to form the YAP–DDX17–p300 complex, which corresponds to increased H3K56 acetylation of stemness-related genes under hypoxia (Figs. 5C and 6D). This concerted regulation of both miRNA biogenesis and histone modification constitutes a unifying mechanism for promoting cancer stem-like features via simultaneously inhibiting the processing of anti-stemness miRNAs and increasing the transcription of stemness-related genes.

Although global downregulation of miRNAs through impaired miRNA biogenesis has been observed in cancer (49), the functional significance of these miRNAs and their relatedness to cancer stemness features are not well characterized. Mori and colleagues have reported 317 miRNAs dysregulated by DDX17 knockdown in HaCaT cells and a sequence motif ([GTA]CATC[CTA]) in the 3’ flanking segment of miRNAs recognized by DDX17 (9). Among the 11 miRNAs we selected that interact with stemness-related genes, 10 of them exhibit this motif, indicating DDX17 may preferentially recognize and bind to a subset of miRNAs. Polyubiquitination of DDX17 at a.a. 190 located between motif-1 and motif-2 inside domain I, which binds to nucleic acids (4), may also disrupt the binding of DDX17 to these miRNAs. Thus, our findings suggest that disruption of DDX17 binding likely represses miRNA biogenesis of anti-stemness miRNAs.

DDX17 is a homolog of DDX5, which also plays a role in miRNA processing (50). However, DDX5 does not have a lysine residue corresponding to K190 on DDX17 that could be ubiquitinated by HectH9. In addition, DDX5 does not interact with YAP (9). Therefore, the signaling pathway regulating DDX5-mediated miRNA processing should be different from the HectH9–DDX17 pathway. A report has shown that p300-mediated acetylation of DDX17, but not of DDX5, contributes to tumorogenesis (51), supporting our observation of the complex formation between p300 and DDX17.

Ubiquitin-binding proteins are responsible for decoding ubiquitin-targeted signal transduction and histone modification. To our best knowledge, this report is the first to demonstrate that a single PTM can negatively and positively modulate two downstream machineries, where both events synergistically increase cancer stem-like properties.

We further show that deposition of H3K56Ac can be found on the promoters of stemness-related genes, particularly BMI1, SOX2, OCT4, along with ubiquitinated DDX17. Hypoxia-induced cooccurrence of DDX17–p300–YAP could H3K56Ac drive the BMI1, SOX2, and OCT4 promoters implies that DDX17 ubiquitination may promote the localization of p300 to the promoters of stemness-related genes and upregulate H3K56 acetylation. However, we could not find any consensus site related to HIF1α or YAP/TEAD binding sites at the regions where DDX17 binds to p2 regions in Bmi1, p3 in Oct4, and p4 in Sox2) using the pair-wise alignment. Therefore, we exclude a putative role for YAP in sequence-specific targeting of the p300/YAP/DDX17 complex. So far, it has not been clearly demonstrated what signaling facilitates H3K56Ac deposition on the promoters of these stemness-related genes, but our results suggest it could be hypoxia. Because embryonic cells also reside in a hypoxic environment (54), our findings suggest that the HectH9–DDX17 axis could act as a driving force to maintain stem cell pluripotency in embryos as well. Our results demonstrate that the HectH9–DDX17 axis could determine cancer stem-like features and that the HectH9-six stemness-related gene (OCT4, SOX2, NANOG, BMI1, NOTCH1, NOTCH2) signature predicts the survival outcome of patients with HNSCC and lung adenocarcinomas.

Our results elucidate the mechanism delineating the connection between global downregulation of miRNAs and tumorigenesis. The concerted regulation of miRNA biogenesis and histone modification by polyubiquitinated DDX17 highlights the importance of blocking DDX17 ubiquitination as a promising therapeutic strategy in cancer treatment. Altogether, we introduce a unifying model posttranslational modification that defines cancer stem-like features. These results will be important both mechanistically and therapeutically for cancer patient management.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Authors’ Contributions

Conception and design: S.-H. Kao, H.-T. Wu, K.-J. Wu
Development of methodology: H.-T. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-H. Kao, Y.-T. Wang, H.-Y. Yeh, Y.-J. Chen
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): W.-G. Cheng, Y.-T. Wang
Writing, review, and/or revision of the manuscript: S.-H. Kao, K.-J. Wu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.-H. Kao, Y.-T. Wang, H.-T. Wu, H.-Y. Yeh, M.-H. Tsai

Study supervision: S.-H. Kao, K.-J. Wu

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Regulation of miRNA Biogenesis and Histone Modification by K63-Polyubiquitininated DDX17 Controls Cancer Stem-like Features

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