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Extrachromosomal circular DNA (eccDNA) undergoes self-aggregation and phase separation with HOXD13 to confer chemotherapy resistance

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Abstract

Extrachromosomal circular DNA (eccDNA) has emerged as a critical regulator driving malignant progression and therapeutic resistance in multiple cancers, yet its molecular mechanism remains poorly defined. Here, we demonstrate that eccDNA could undergo self-aggregation *in vivo* and *in vitro*, and interacted with HOXD13 protein. EccDNA promotes Esophageal Squamous Cell Carcinoma (ESCC) cell proliferation, migration, and cisplatin resistance by modulating HOXD13 condensate dynamics. Ectopic delivery of eccDNA restored cisplatin resistance in HOXD13-knockout ESCC cells, while depletion of HOXD13 abolished this phenotype. Functional assays including CCK-8, EDU incorporation and Transwell assays revealed that silencing HOXD13 markedly suppressed ESCC cell proliferation, DNA synthesis, and migratory capacity *in vitro*. Mechanistically, eccDNA or chemical cross-linking agents induced the formation of HOXD13 condensates, which distributed either the nucleus or cytoplasm. Screening of nucleocytoplasmic transport factors identified KPNB1 as the key importin mediating HOXD13 nuclear shuttling. Transcriptomic and CUT & Tag analysis revealed that eccDNA increased the immune response pathway and cancer signaling. In addition, we showed that eccDNA promoted the interaction of HOXD13 and NFκB. In xenograft mouse models, combined treatment with DNase I-mediated eccDNA clearance and cisplatin synergistically restrained tumor growth and elevated DNA damage compared with monotherapy. Collectively, our findings reveal a novel eccDNA-HOXD13 pathway that modulates ESCC malignancy and chemoresistance, highlighting eccDNA clearance as a promising strategy to improve the therapeutic efficacy of cisplatin in ESCC.

Key Words: Extrachromosomal circular DNA (eccDNA), HOXD13, Chemoresistance

Introduction

Extrachromosomal circular DNA contributes to powerful roles in evolution and in cancer initiation and progression and drug resistance during cancer treatment by driving genetic plasticity and heterogeneity from extrachromosomal sites[1]. Cancer cells harbor heterogeneous populations of ecDNAs with various sizes. EcDNA refers to circular DNA molecules that are self-replicating, clonally selected and amplified and range from 100 kilobases (kb) to several megabases (Mb) in size. eccDNA is classified as smaller, nonclonal, extrachromosomal elements [2, 3]. Early studies suggest eccDNA arises when DNA breaks and is subsequently repaired incorrectly, with key mechanisms including nonhomologous end joining, alternative end joining, and homologous recombination[4], indicating eccDNA may derive from all parts of their genome. EccDNA-borne genes were often accompanied by increased expression, and their contribution to expression was much larger than that from linear amplifications. The cytokine gene CXCL5 exemplified this phenomenon, showing substantial copy-number increase and upregulation when present on eccDNA[5]. EccDNA also has immunological roles — research has found that eccDNAs are apoptotic products with high innate immunostimulatory activity, linking them to immune surveillance mechanisms[6]. However, the molecular mechanisms by which the cell recognizes or processes these ecDNAs remain elusive.

Liquid-liquid phase separation (LLPS) is the process by which biomolecules — particularly proteins with intrinsically disordered regions (IDRs) — demix from the surrounding nucleoplasm to form dynamic, membraneless condensates. Unlike solid–liquid phase separation, LLPS is highly dynamic and reversible, enabling the spatial and temporal organization of cellular components without the need for lipid membranes. This process is driven by weak, multivalent intermolecular forces, including electrostatic interactions, hydrogen bonding, and van der Waals forces[7]. The intrinsically disordered regions (IDRs) of proteins play a particularly important role in mediating LLPS, as they facilitate the multivalent interactions necessary for condensate formation. Nucleic acids, especially RNA, can further promote LLPS by electrostatically interacting with protein IDRs. Coactivators BRD4 and MED1 form liquid-like nuclear puncta via LLPS in super-enhancer (SE) regions, enhancing the expression of key genes[8].The connection between ecDNA and phase separation is that ecDNA hubs are an integrated part of nuclear condensates and exhibit cancer-type-

specific chromatin connectivity[9]. However, the relationship between small eccDNA and phase separation is unclear.

HOXD13 (Homeobox D13) encodes the homeobox protein, a member of the homeobox family of genes, which encode a highly conserved family of transcription factors to regulate morphogenesis in all multicellular organisms. The inactivation of the Hoxd13 gene in mice causes a phenotype resulting from defects in the proliferation and/or condensation of limb mesenchymal cells[10]. Dysregulated HOXD13 expression has been detected in breast cancer, melanoma, colon cancer, cervical cancer, and astrocytomas, with the majority of cancers showing an increase in HOXD13 expression [11, 12]. Mechanistically, HOXD13 was shown to bind the PTPRN2 promoter and promote its transcription, suggesting HOXD13 may be a candidate marker for the diagnosis and treatment of colon cancer[12]. The N terminal intrinsically disordered regions (IDRs) stimulates HOXD13 to undergo LLPS to regulate transcription, while is disrupted by abnormal repeat expansions in some human disorders[13]. Improved understanding of the regulation of HOXD13 condensate formation is beneficial for cancer therapy.

The findings establish eccDNA as a potential biomarker for tumor diagnosis and prognostic assessment. Results linking eccDNA to oncogene amplification and drug resistance have highlighted possible uses of eccDNA in tumor therapy and monitoring, including in gliomas, gynecologic tumors, and genitourinary disorders. In summary, small eccDNAs — once considered genomic curiosities — are now understood as dynamic, functionally potent molecules with roles spanning normal development, aging, immunity, and cancer biology. Their unique circular topology, open chromatin state, and capacity for autonomous amplification make them a frontier of both basic and translational research.

Results

EccDNA increases in chemotherapy resistant tumor

To study the relationship between eccDNA and chemotherapy resistance, total eccDNAs were extracted from cisplatin resistance cells following reported protocol (Fig. 1A), with higher level in cisplatin resistant stable cell lines A549R and KYSE150R compared to parental cells (Fig. 1B). Consistently, the content of eccDNA was higher in cisplatin resistant esophageal squamous cell carcinoma (ESCC) samples than sensitive counterpart (Fig. 1C). To investigate the underlying mechanism of

ecDNA in the progress of cisplatin resistance, we labeled purified eccDNA with biotin. As a circular ecDNA, we introduced a single strand nick by enzyme FnoCas12a (Cpf1) in the absence of guide RNA (Fig. 1D) [6]. The enzyme treatment did not change the main size and appearance as shown in agarose electrophoresis (Fig. 1E). After delivering biotin-eccDNA into cells using standard transfection, successful biotin labeling was revealed by immunofluorescence by streptavidin-FITC antibody (Fig. 1F). Next, we using eccDNA with biotin tag to perform a pulldown assay with tissue lysate, which were subsequently analyzed by mass spectrum. Among significant enriched overlap genes from mass spectrum assay and RNA-seq were HOXD13, XCRR5, MCM6, PRKDC, PARP1 and so on (Fig. 1G), of which protein MCM6, PARP1, PRKDC have been identified recently by other independent groups [14]. The enriched proteins were subsequently used to perform GO analysis, which revealed that eccDNA participates various biological regulation events related to DNA, like catalytic activity ancting on DNA, single stranded DNA binding, damage DNA binding and so on (Fig. 1H). Taken together, the data showed that the level of eccDNA increases in chemotherapy resistant samples and cells, potentially by interacting with key proteins.

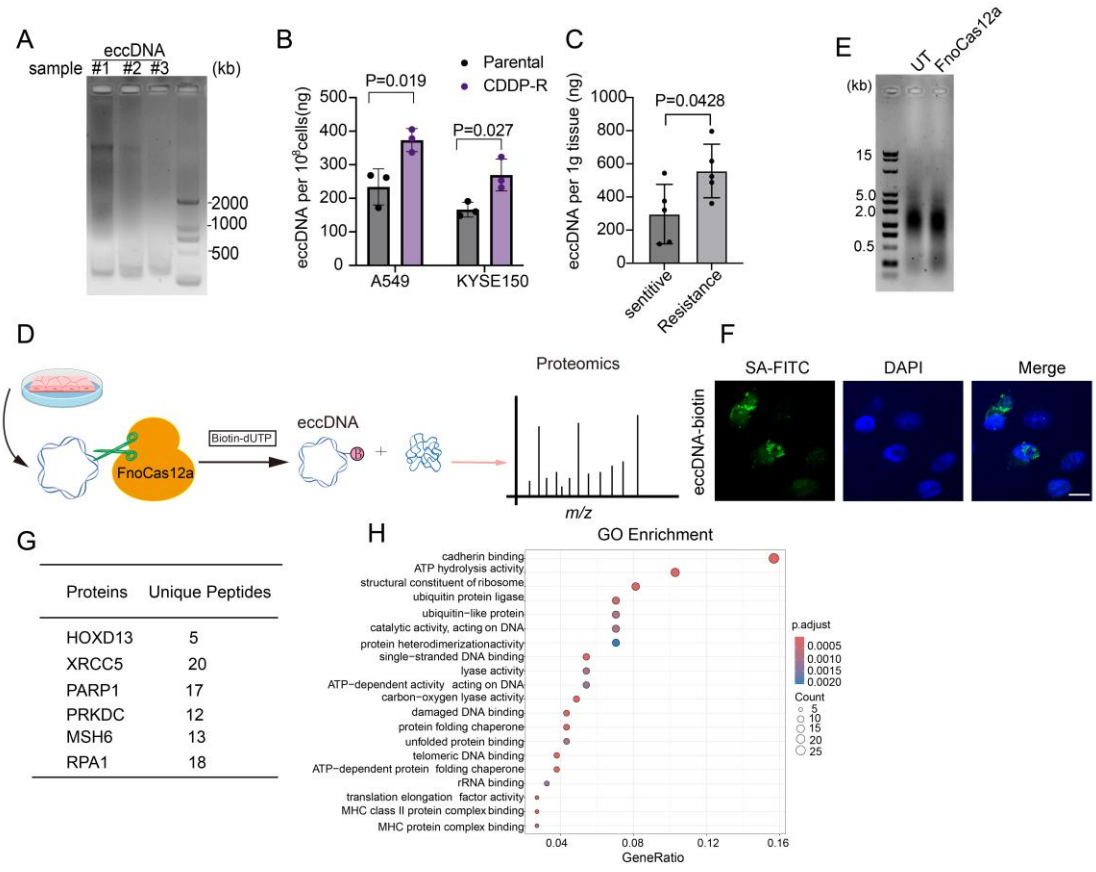


Figure 1. eccDNA is associated with chemotherapy resistance. (**A**) Representative image of gel electrophoresis of eccDNA. (**B-C**) quantification of eccDNA content in CDDP resistant cell line and chemotherapy resistance clinical samples. (**D**) Diagram depicting RNA sequencing clinical samples of cisplatin resistant and sensitive ESCCs (n=10). EccDNA were extracted and cut by FnoCas12a (Cpf1) (100nM) to produce one nick, and labeled with biotin-11-dUTP by Terminal Deoxynucleotidyl Transferase. The anti-biotin-ecDNA immunoprecipitates was analyzed by mass spectra. (**E**) Representative image of gel electrophoresis of eccDNA treated with FnoCas12a (Cpf1). (**F**) Representative immunofluorescence images of biotin-eccDNA with streptavidin-FITC (SA-FITC) antibody. Scale bar: 10μm. (**G**) Enriched genes identified from biotin-eccDNA pulldown. (**H**) GO term enrichment analysis enriched genes.

EccDNA is prone to aggregate and promotes liquid-liquid phase separation

We speculate that whether phase separation of HOXD13 links cisplatin resistance and ecDNA. By stained HOXD13 with fluorescence conjugated antibody, we unexpected discovered treating ESCC cell lines with cisplatin instead of 5-fluorouracil or ecDNA induce the obvious formation of HOXD13 condensates. Such HOXD13 condensates were shown to be the liquid-liquid droplet as treatment with 1,6-hexanediol diminished condensate. Moreover, DNase I treatment reduced HOXD13 condensates, indicating ecDNA is pivotal for condensate formation (Fig. 2A). To observe directly, we synthesized DNA to mimic ecDNA and labeled it with fluorescent dye, and delivered it into cells. As expected, most of HOXD13 condensates were found in cojunction with ecDNA foci (Fig. 2B). We noted that some cell lines like ESCC derived cell line KYSE30 and KYSE 450 did not develop HOXD13 condensate unless be induced by some chemicals such as cisplatin, camptothecin, acrolein (smoking byproduct in cell) and alcohol (Fig. 2A), which were thought to cause the cross-link of cellular DNA including ecDNA. Therefore, we reason that cross-link ecDNA may enhance the formation of HOXD13 condensate. To test this, ecDNAs were isolated and reacted with cisplatin, acrolein and acetaldehyde (AcH) in tube, then be incubated with recombinant HOXD13-IDR-mcherry protein (Fig. 2C). As expected, the formation of HOXD13 condensate increased much more remarkable after incubating with eccDNA, CDDP-DNA, AcH-DNA or Acrolein-DNA (Fig. 2D). Using Electrophoretic Mobility Shift Assay (EMSA), we demonstrated that extrachromosomal DNA bound HOXD13 protein *in vitro*, which was enhanced by cisplatin treatment (Fig. 2E). Furthermore, we performed phase separation of HOXD13 in vitro by incubating purified HOXD13-IDR-

mcherry protein and various concentration of ecDNA. The result demonstrated that ecDNA with high concentration enhanced the phase-separation capacity of the HOXD13 IDR (Fig. 2F). These data imply that the dosage of ecDNAs is critical the formation of HOXD13 condensate. The dynamic property of biocondensate formation resembled the transient expression burst observed during the exogenous plasmid expression process, suggesting that this burst may contribute to HOXD13 condensate formation. Taken together, these findings strongly indicate that local aggregation and secondary structure of ecDNA are critical drivers for HOXD13 condensate formation.

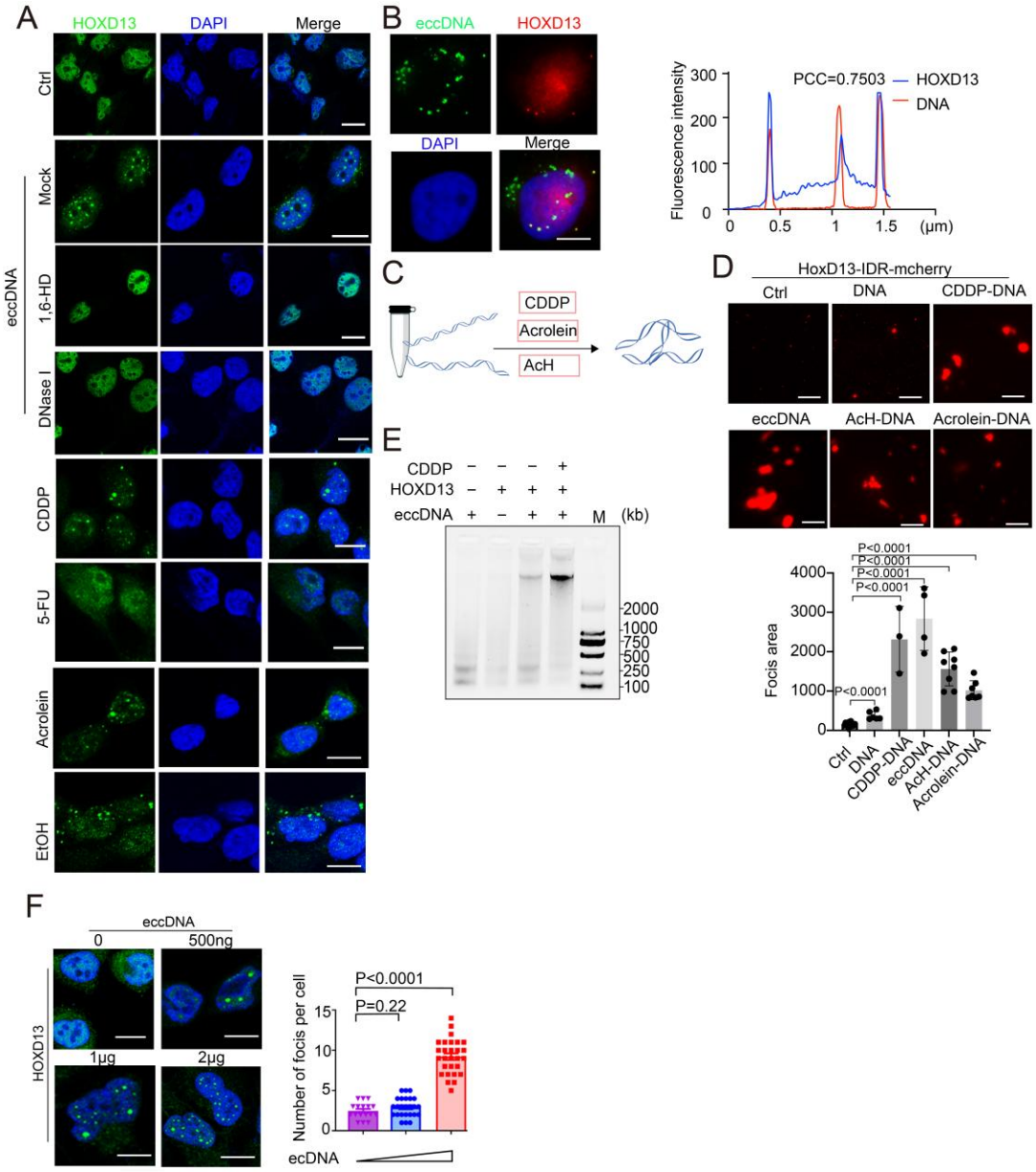


Figure 2. eccDNA undergoes aggregation and phase separation. (**A**) Representative immunofluorescence images of HOXD13 condensates in KYSE30 cells treated with eccDNA, cisplatin (5μM, 24h), 5-FU (10μM, 24h), ethanol (5μM, 24h), acrolein (20μM) and 1,6-hexanediol. Scale bar: 10μm. HOXD13 droplets per cells were quantified. (**B**) Representative immunofluorescence images of HOXD13 condensates and eccDNA-Biotin. Co-localization analysis was performed by Image J. Pearson correlation coefficient (PCC) is 0.7503. (**C**) Diagram depicting cross-link DNA. (**D**) LLPS of GST-HOXD13-mcherry mixed with DNA, eccDNAs and genomic DNA treated with cisplatin, acrolein and acetaldehyde *in vitro*. Scale bar: 20μm. (**E**) Electrophoretic mobility shift assay of recombinant HOXD13 protein (5μM) incubated with ecDNA (50ng/μl) *in vitro*. (**F**) Representative immunofluorescence images of HOXD13 condensates in KYSE30 cells after transfection of eccDNA as indicated. Scale bar: 10μm.

EccDNA enhances cancer proliferation and migration

To explore the biological function of eccDNA during the malignant progression of esophageal squamous cell carcinoma (ESCC), we delivered eccDNA in two ESCC cell models, KYSE30 and KYSE150. Cell proliferative activity was then measured using the CCK-8 assay, which demonstrated that eccDNA significantly promoted the growth rate in both KYSE150 and KYSE30 cells (Fig. 3A). An EDU incorporation assay further indicated that eccDNA stimulated the efficiency of DNA synthesis within the cell division cycle (Fig. 3B). Beyond cellular proliferation, we also examined the influence of HOXD13 on ESCC cell migratory behavior. Transwell migration assays consistently revealed that eccDNA markedly promoted the migration ability of KYSE30 and KYSE150 cells (Fig. 3C). Moreover, by delivering eccDNA into HOXD13 KO cells, we found that eccDNA promoted cisplatin resistance, whereas knocking out HOXD13 abolished its effects (Fig. 3D). Collectively, these in vitro findings demonstrate that eccDNA enhances the proliferation and migration of ESCC cells mainly through HOXD13.

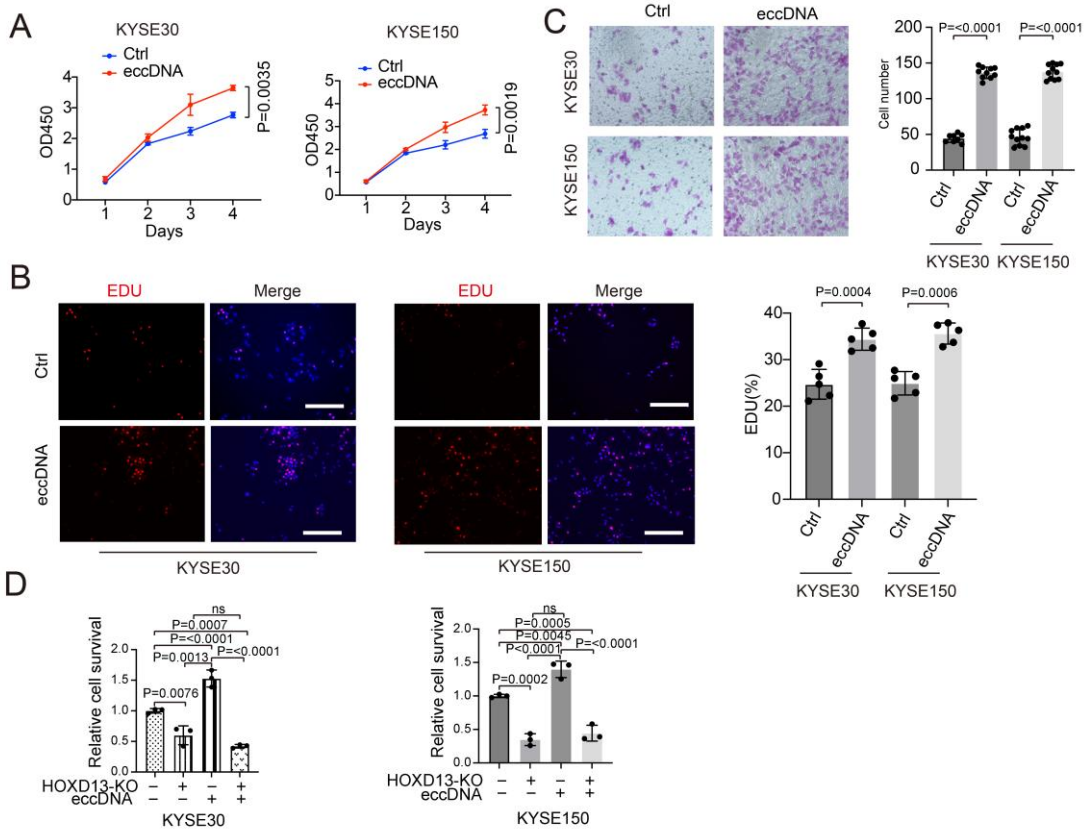


Figure 3 eccDNA promotes ESCC proliferation and migration. (**A**) Cell proliferation assay of KYSE150 and KYSE30 cells transfected with eccDNA, with each counting performed in triplicate. (**B**) EdU incorporation assay of KYSE150 and KYSE30 cells transfected with eccDNA. Scale bar: 50 μm. (**C**)Transwell assay comparing KYSE30 and KYSE150 cells transfected with eccDNA and control. (**D**) Relative cell survival rate of HOXD13 KO KYSE30 and KYSE150 cells treated with cisplatin (10μM) for 48h after transfection with eccDNA.

KPNB1 regulates HOXD13 condensate nuclear importation

Our data showed that HOXD13 condensates induced by ecDNA or cross-link drugs located either in nuclear or cytoplasm (Fig.2A), indicating HOXD13 condensate may reside both in the nuclear or cytoplasm, due to nucleotides aggregate in some circumstance. To test this possibility, we used cycloheximide (CHX), a translation elongation inhibitor, to block mRNA processing in ribosomes, thereby creating a local burst of mRNA in the cytoplasm [15]. As expected, CHX treatment induced the formation of HOXD13 condensates in the cytoplasm both in KYSE 30 and KYSE 150 cell lines (Fig. 4A). To find out the nuclear transporting protein, we tried to screen the interacting proteins of HOXD13 by mass spectrometry. The anti-HOXD13 immunoprecipitates were isolated from cells treated with cisplatin and control. Then,

we analyzed the enriched interacting protein of HOXD13 by online database STRING. DNA damage response and nucleocytoplasmic transport proteins were selected for network assay (Fig. 4B and 4C). Karyopherin-β (Kap) is responsible for the majority of proteins shuttling from cytoplasm to nucleus. By knocking down the importins candidates by siRNA transfection, we identified that KPNB1 is required for nuclear location of HOXD13 condensates rather than KPNA2 and KPNA3 (Fig. 4D). The knocking down efficiency were verified by WB (Fig. 4E). In addition, we demonstrated that knocking down KPNB1 diminished the nuclear location of HOXD13, while overexpressing KPNB1 promoted the nuclear location of HOXD13 (Fig. 4F). However, knocking down KPNB1did not affect the nuclear location of eccDNA (Fig.4G). To summary, KPNB1 modulate the nuclear location of HOXD13 condensate via mediating nucleocytoplasmic transport of HOXD13 proteins.

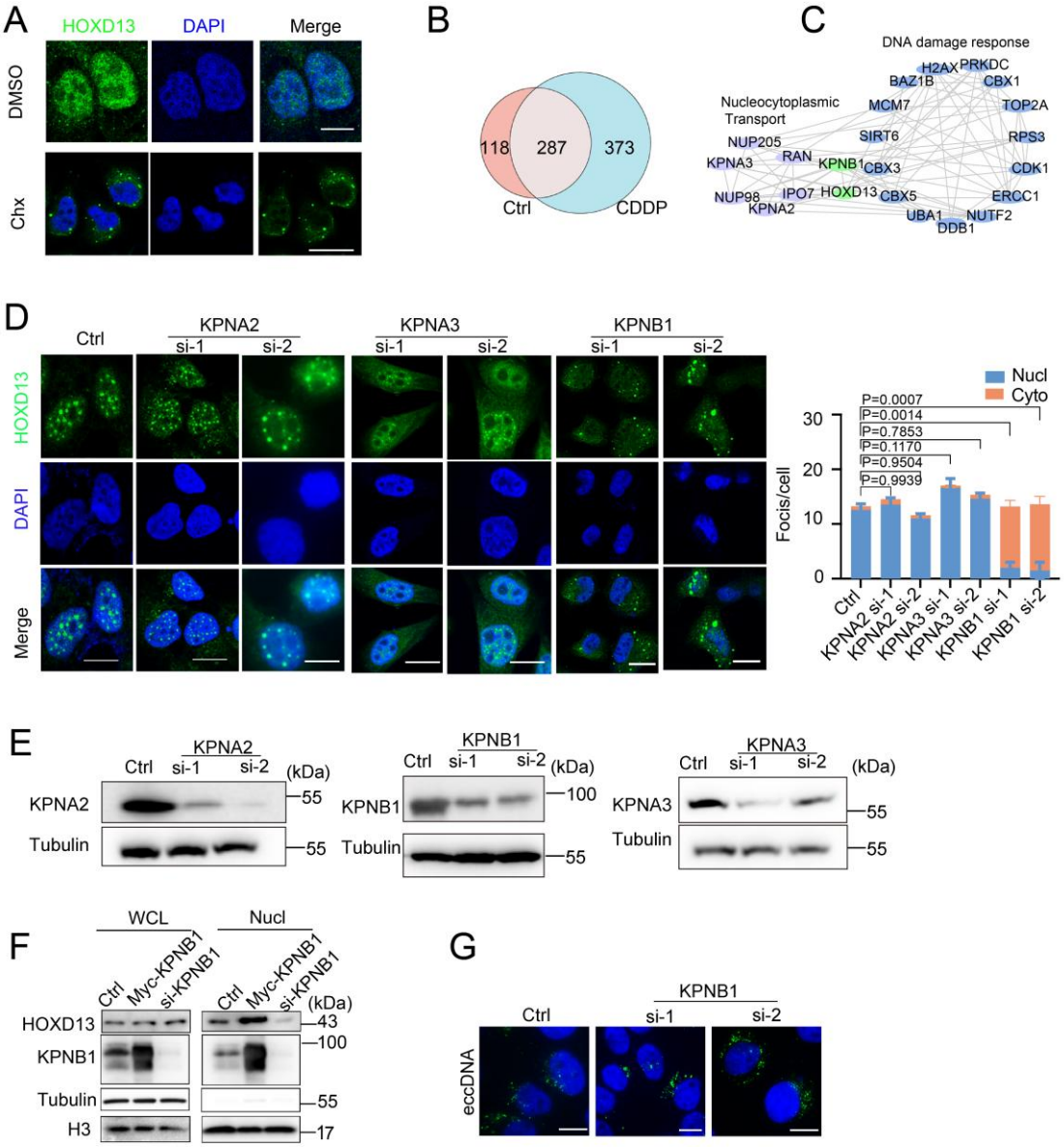


Figure 4 KPNB1 promotes HOXD13 condensate nuclear location. (**A**) Representative immunofluorescence images of HOXD13 condensates in KYSE150 cells treated with cycloheximide (Chx, 50μg/ml). Scale bar: 10μm. **(B)** Enriched genes of mass spectrometry of anti-FLAG-HOXD13 immunoprecipitates in cisplatin treated cells. (**C**) GO analysis of HOXD13-interacting proteins after CDDP treatment based on database STRING. (**D**)Representative immunofluorescence images of HOXD13 condensates in cells after knocking down KPNA2, KPNA3 and KPNB1. (**E**) Immunoblots in cells transfected with KPNA2, KPNA3 and KPNB1 siRNA as indicated. (**F**) Western blotting analysis of HOXD13 in whole cell lysates (WCL) and nuclear fractions (Nucl) after knocking down or overexpressing KPNB1. (**G**) Representative immunofluorescence images of biotin-eccDNA after knocking down KPNB1. Scale bar: 10μm. *P* values in (**D**) were calculated by one-way ANOVA with multiple comparisons.

EccDNA links immunity and cancer pathway by regulating NF-κB

Previous study reported that eccDNA stimulated innate immunity via cGAS-STING, while its role in cancer progression is largely unknown. The HOXD13 condensate is shown to be associated with H3K27ac modification to regulate transcription process[13]. To investigate the underlining mechanism, we transfected cells with exogeneous eccDNA, which were further subjected to transcriptomic and H3K27ac CUT & Tag analysis (Fig.5A). RNA sequencing data revealed that substantial genes including genes related to immunity response like CXCL13, CCR9 and CD1A were significantly upregulated upon eccDNA treatment (Fig.5B). Consistently, GO analysis showed that immune and inflammatory response pathways were highly enriched (Fig.5C). Preliminary data showed that eccDNA interacts with HOXD13, which is considering to guild the transcription process via H3K27 acetylation. We then performed the CUT & Tag experiment with H3K27ac antibody, which implied that eccDNA stimulated the transcription of H3K27ac enriched genes (Fig.5D). GO analysis revealed that GTPase activity pathway were highly enriched in eccDNA treated cells (Fig.5E), and the enriched peaks located in the promoter regions (Fig.5F). In addition, several common genes such as DACT1, WWC1, CD83 and DDX11 were identified in both RNA-seq and H3K27ac CUT&Tag analysis, which were annotated as regulating genes in immune response and cancer progression (Fig.5G, 5H). To further explore the molecular mechanism, we noticed that NF-κB, a master regulator in inflammatory response, interacted with HOXD13 in mass spectral data. Co-IP experiment demonstrated that eccDNA enhance the interaction between HOXD13 and NF-κB (Fig.5I), indicating that eccDNA may stimulate the cooperation of HOXD13 and NF-κB in guiding the transcription process.

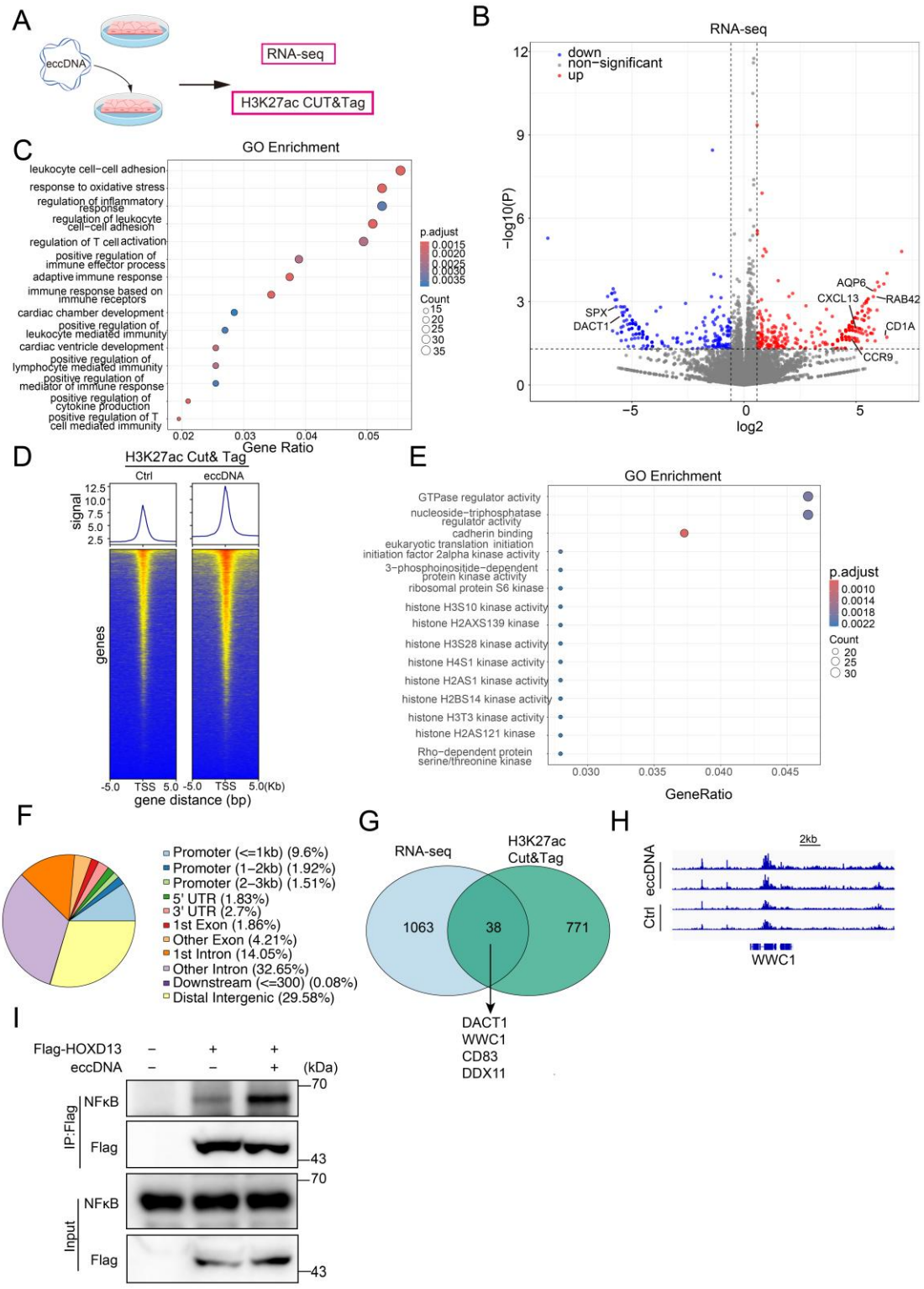


Figure 5 eccDNA promotes transcription. (**A**) Diagram depicting RNA sequencing and H3K27ac CUT&Tag analysis of cells transfected with eccDNA. (**B**) Volcano plot of gene expression levels based on transcriptomic data in eccDNA overexpressing cells. (**C**) GO term enrichment analysis based on transcriptomic data. (**D**) CUT&Tag sequencing was performed using a H3K27ac antibody.

The TSS heatmap shows gene transcription levels in cells treated with eccDNA. (**E**) GO term enrichment analysis based on transcriptomic data. (**F**) Pie chart illustrating the percentage of genomic occupancy for H3K27ac bound peaks. (**G**) Venn diagram depicting overlapping genes between RNA sequencing and CUT&Tag. (**H**) CUT&Tag sequencing analysis of the read density of the WWC1 transcript after H3K27ac antibody treatment with ecDNA. (**I**) NFκB immunoblots in anti-FLAG immunoprecipitates in HEK293T cells transfected with FLAG-HOXD13.

Clearance of eccDNA enhances chemotherapy sensitivity

To test whether reducing the eccDNA could sensitize cisplatin therapy, we treated cisplatin resistant cell lines with DNase I and cisplatin. Our results demonstrated that co-treatment with DNase I and cisplatin significantly improved cisplatin responsiveness in these resistant cells (Fig.6A). To assess the therapeutic efficacy in living organisms, we administered DNaseI and cisplatin in subcutaneous nude mouse xenograft tumor model. Consistent with our hypothesis, combined administration of DNaseI and cisplatin effectively suppressed tumor development (Fig. 6B-D). Given that cisplatin exerts its antitumor effects by inducing DNA damage and apoptotic cell death, we performed γH2AX staining on tumor tissues isolated from various treatment groups. The resulting data demonstrated that tumors from animals receiving both DNaseI and cisplatin exhibited the most pronounced levels of DNA damage and apoptosis (Fig. 6E and 6F). Taken together, partial elimination of eccDNA by DNase I is beneficial for improve cisplatin therapy efficiency.

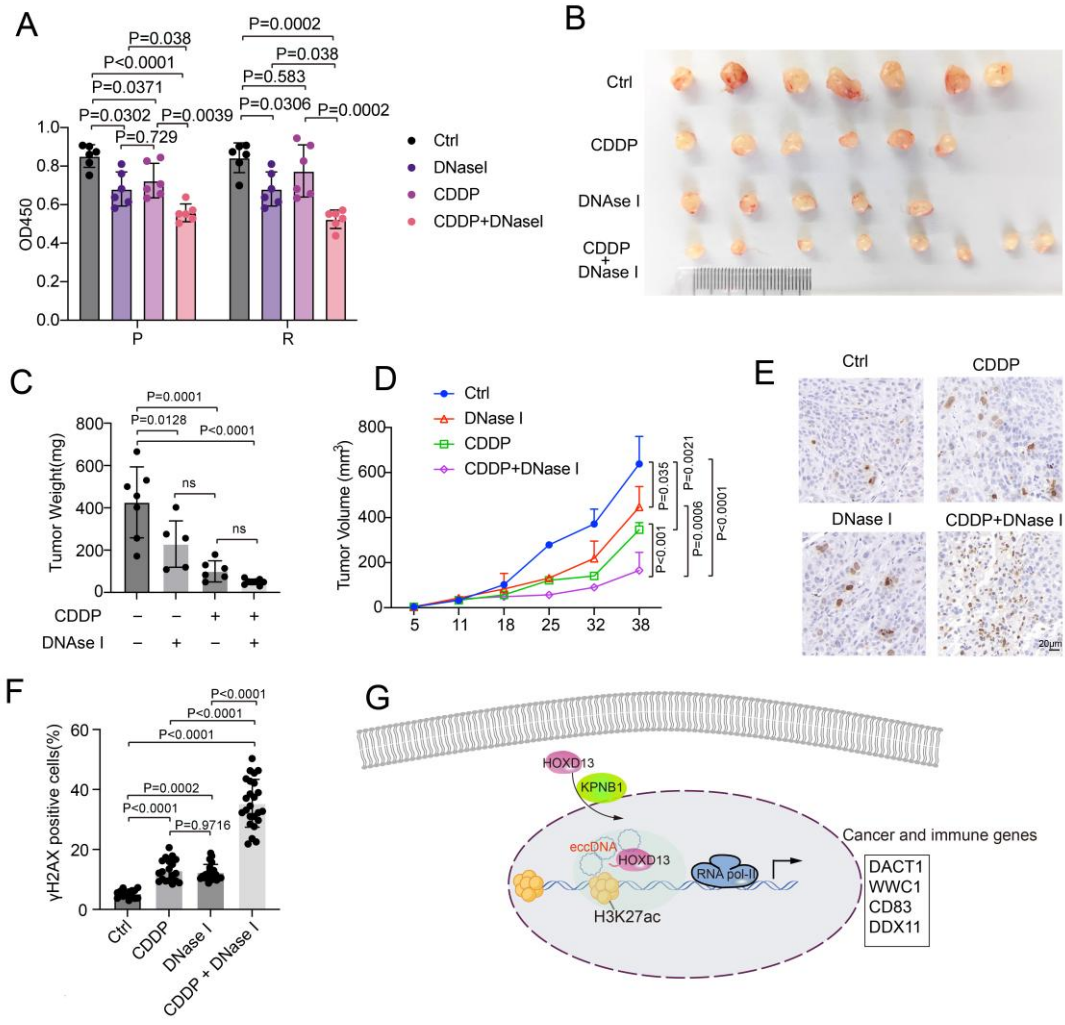


Figure 6 elimination of eccDNA sensitizes chemotherapy. (**A**) Cell proliferation assay of CDDP resistant and parental cells treated with CDDP and/or DNase I. (**B-D**) Representative images, tumor volume and size showing subcutaneous xenograft model treated with CDDP and/or DNase I. (**E-F**) Immunological staining was used to detect γ-H2AX was performed in tumor samples. **(G)** Working model. *P* values in (**A, C, F**) were calculated by one-way ANOVA with multiple comparisons.

Discussion

Cancer patients experience cycles of disease relapse and retreatment, eventually developing progressive, chemoresistant tumors[16]. In this study, we delineate a previously unrecognized molecular axis through which eccDNA drives chemotherapy resistance and malignant progression in esophageal squamous cell carcinoma (ESCC). Our findings demonstrate that eccDNA promotes cisplatin resistance via HOXD13

condensate formation, nuclear translocation mediated KPNB1, and that pharmacological clearance of eccDNA using DNaseI restores chemosensitivity *in vivo*. These results unravel the novel molecular mechanism of the recognition of eccDNA by LLPS and the cytonuclear transport mediated by KPNB1(Fig.6G).

Extrachromosomal circular DNA has garnered considerable attention as a non-canonical amplification mechanism that enables rapid oncogene amplification and intratumoral heterogeneity, circumventing the constraints of linear chromosomal inheritance [17, 18]. Unlike chromosomally integrated amplicons, eccDNA lacks centromeres and replicates autonomously, allowing dynamic copy number fluctuations that accelerate clonal evolution under therapeutic pressure[19]. Consistent with these concepts, our data show that eccDNA delivery directly confers cisplatin resistance in ESCC cells, extending prior observations in glioblastoma and other solid tumors where eccDNA-mediated amplification of resistance genes — such as *EGFR* and *MYC* — enables rapid adaptation to targeted therapies [20]. Importantly, our study identifies HOXD13 as a downstream transcriptional effector of eccDNA in ESCC, providing a mechanistic link between eccDNA burden and the transcriptional reprogramming that sustains tumor cell survival under genotoxic stress.

HOX family transcription factors are well-established regulators of developmental patterning, and their dysregulation has been increasingly implicated in oncogenesis across multiple cancer types [21]. HOXD13, in particular, has been reported to promote cell proliferation, epithelial-mesenchymal transition, and metastasis in several malignancies, including breast and colorectal cancers [12]. Our results reinforce and extend these findings to ESCC, demonstrating that HOXD13 is indispensable for eccDNA-driven proliferative and migratory phenotypes. The significant attenuation of clonogenic capacity, DNA synthesis, and cell migration upon HOXD13 knockdown underscores its multifaceted role in sustaining the malignant phenotype. Furthermore, the observation that HOXD13 forms biomolecular condensates in response to genotoxic stimuli adds a new layer of regulatory complexity to HOX transcription factor biology, suggesting that their oncogenic functions may be spatiotemporally regulated through phase separation mechanisms.

The concept of biomolecular condensates — membrane-less organelles formed through liquid-liquid phase separation (LLPS) — has transformed our understanding of gene regulation and stress response [22, 23]. Transcription factors and co-activators enriched in intrinsically disordered regions (IDRs) are now recognized to concentrate

at super-enhancers through phase separation, amplifying transcriptional output in cancer cells [24]. Stress granules and other cytoplasmic condensates similarly form in response to diverse cellular insults, sequestering mRNAs and signaling molecules to modulate the cellular stress response [25]. Our finding that HOXD13 condensates form in the cytoplasm upon translational stress — induced by cycloheximide or excessive nucleotide burden and subsequently translocate to the nucleus to drive transcriptional programs is consistent with emerging evidence that condensate dynamics are a key regulatory node in cancer biology [26]. The subcellular partitioning of such condensates may thus serve as a rheostat, calibrating transcriptional output in response to the tumor microenvironment and chemotherapeutic pressure. KPNB1 (importin-β1), the canonical import receptor, recognizes NLS-containing cargo either directly or via adaptor importin-α subunits, and transports them through nuclear pore complexes in a Ran-GTPase-dependent manner[27]. Our identification of KPNB1 as the requisite importin for HOXD13 condensate nuclear translocation — and the dependence of this places nucleocytoplasmic transport as a central regulatory event downstream of eccDNA signaling.

The therapeutic implications of our findings are significant. DNase I-mediated degradation of extracellular and intracellular DNA has been explored in the context of autoimmune diseases and NETs (neutrophil extracellular traps) [28], but its application to directly ablate eccDNA as a cancer therapy represents a conceptually novel approach. Our PDX experiments demonstrate that combining DNase I with cisplatin produces synergistic anti-tumor effects, accompanied by elevated DNA damage markers (γH2AX), consistent with the idea that eccDNA clearance dismantles the transcriptional programs that sustain cisplatin resistance. These findings complement recent strategies targeting eccDNA biogenesis and maintenance, including inhibition of topoisomerase II and DNA repair pathways implicated in eccDNA formation. Future studies should explore the pharmacokinetics and tumor penetration of DNase I delivery systems, as well as potential combinatorial strategies with immune checkpoint blockade, given the known immunostimulatory effects of extrachromosomal DNA through cGAS-STING pathway activation[29, 30].

Several important questions remain to be addressed. First, the precise structural features of HOXD13 that drive condensate formation — particularly the role of its IDRs and homeobox domain — merit detailed biophysical characterization. Second, while we demonstrate that KPNB1 is required for HOXD13 nuclear import, the specific

nuclear localization signal (NLS) recognized by this importin remains to be mapped. Third, the upstream mechanisms by which eccDNA triggers HOXD13 condensate nucleation, whether through direct transcriptional upregulation, post-translational modifications, or changes in the local nucleotide abundance, need further investigation. Finally, the generalizability of the eccDNA–HOXD13 pathway to other cancer types should be systematically evaluated, as eccDNA has been detected across a broad spectrum of human malignancies[31].

Conclusion

In summary, our study establishes a mechanistic framework in which eccDNA is prone to aggregate and condensates with HOXD13 to drive transcriptional reprogramming, cisplatin resistance, and tumor progression in ESCC. These findings not only advance our fundamental understanding of eccDNA biology but also nominate the components of this pathway — particularly KPNB1-mediated nuclear import and eccDNA itself — as actionable therapeutic targets. Targeting eccDNA clearance in combination with standard chemotherapy represents a promising and clinically translatable strategy to overcome drug resistance in ESCC and potentially other eccDNA-driven malignancies.

MATERIALS AND METHODS

Human Tissues and Cell Lines

For eccDNA extraction, 5 fresh ESCC tumor tissues were collected. Written informed consent was acquired from every enrolled patient, and the research protocol was approved by the Research Ethics Committee of the Cancer Hospital of Tianjin Medical University (Approval batch: bc2021340). Cell lines including HEK293T, KYSE30, KYSE150 and HeLa were purchased from the American Type Culture Collection and cultured in strict accordance with the supplier's instructions. All cell lines were maintained in medium recommended by Life Technologies (USA), supplemented with 10% fetal bovine serum and a mixed solution of penicillin and streptomycin at a concentration of 100 U/mL. Cells were incubated under standard conditions: 37°C, 5% CO_2 and atmospheric oxygen tension. Short tandem repeat (STR) genotyping was applied to authenticate all cell lines. To ensure cell line purity and stability, routine mycoplasma detection was performed monthly to exclude contamination.

Plasmid Construction

The full-length complementary DNA (cDNA) encoding human HOXD13 was

amplified from total RNA isolated from HeLa cells, then inserted into the pcDNA3.1 vector fused with FLAG epitope tags (Invitrogen, USA). The pHR-HOXD13-IDR-mCherry-CRY2 plasmid was acquired from Addgene (catalog #145258), and the HOXD13-IDR-mCherry segment was subcloned into the pGEX-4T3 vector (GE Healthcare Life Sciences) for recombinant protein purification. A truncated HOXD13 construct lacking amino acid residues 1–175 at the N-terminus was amplified and cloned into the pKH3HA vector (Addgene). All newly constructed plasmids were verified by DNA sequencing.

RNA Interference

Small interfering RNAs (siRNAs) targeting HOXD13, KPNA2, KPNA3 and KPNB1 were chemically synthesized and introduced into cultured cells via Lipofectamine 3000 transfection reagent, following the manufacturer's standard protocol. The oligonucleotide sequences for siRNAs are listed in Table1. For transfection, 100 pmol of each siRNA was combined with 5 μL of Lipofectamine 3000 reagent, and the mixture was incubated at room temperature for 5 minutes before being added to cells seeded in 12-well culture plates. Cells were collected 48 hours after transfection, and corresponding protein expression levels were analyzed by Western blotting (WB).

Isolation of eccDNA and labeling with biotin

The protocol used for the isolation of eccDNA followed a previous report with modifications [32]. Briefly, confluent cells were harvested and fixed with 95% methanol. The cells were lysed with alkaline lysis buffer with RNase A at pH 11.8 (Qiagen Kit). After neutralization and precipitation (buffer S3 from the Qiagen Kit), crude eccDNA was mixed with binding buffer (buffer BB) to bind a silica column. Bound eccDNA was then eluted and digested with PacI (NEB) and Plasmid-Safe ATP-dependent DNase (Lucigen) for 4–16 h, extracted with a PCR purification kit (Qiagen) and quantified with a Qubit dsDNA HS Assay kit.

The purified eccDNA was cut by 100 nM FnoCas12a (Cpf1) (NEB) to introduce one nick per circular DNA in the reaction buffer (10 mM MnCl2, 10 mM Tris-HCl) in the absence of guide RNA [28]. EccDNA was then purified and labeled with biotin-11-dUTP by terminal deoxynucleotidyl transferase (Beyotime Biotechnology) following the manufacturer's instructions. eccDNA was transfected into cells with Lipofectamine 3000 (Invitrogen) in Opti-MEM (Gibco) according to the manufacturer's instructions.

The transfection efficiency was determined by immunofluorescence using a streptavidin-FITC antibody (Beyotime Biotechnology).

Immunoprecipitation

HEK293T cells were transfected with the indicated plasmids and collected 48 hours after transfection. Cell lysis was carried out on ice for 30 minutes using lysis buffer containing 50 mM Tris-HCl, 250 mM NaCl, 0.2% NP40, 10% glycerol, and protease inhibitors. After brief sonication and centrifugation, the supernatant was collected and incubated with specific antibodies overnight at 4°C. Protein A/G magnetic beads were then added and incubated for an additional 1 hour at 4°C. Beads were washed three times to eliminate non-specific binding, then heated in SDS sample buffer for 5 minutes. The eluted proteins were resolved by SDS-PAGE, transferred to membranes, and detected by Western blotting (WB) using the specified antibodies.

Immunofluorescence Staining

Cells were plated on sterile coverslips placed in 12-well plates and incubated overnight. After the designated experimental treatment, cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes and rinsed three times with phosphate-buffered saline (PBS). Cells were then permeabilized using 0.25% Tween-20 in PBS for 10 minutes, followed by blocking with 5% bovine serum albumin (BSA) for 1 hour. Samples were then incubated overnight at 4°C with primary antibodies of interest. After three washes, cells were incubated with fluorophore-conjugated secondary antibodies and mounted using ProLong Gold mounting medium (Invitrogen). Fluorescent images were captured using a laser scanning confocal microscope (LSM 800, ZEISS).

Immunohistochemistry

Immunohistochemical staining was carried out according to a previously published protocol with slight modifications [57]. Tissue specimens were fixed in 4% paraformaldehyde (PFA) at room temperature for 10 minutes and then embedded in paraffin. Paraffin sections were deparaffinized and dehydrated, followed by antigen retrieval performed in 10 mM citrate buffer at 70°C for 20 minutes. To block non-specific immunoreactivity, sections were incubated with a blocking solution containing 5% donkey serum and 1% bovine serum albumin (BSA) for 30 minutes. Sections were then incubated overnight at 4°C with an anti-HOXD13 primary antibody (1:100;

Abcam). After washing with PBS, sections were treated with a horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody for 30 minutes. Signals were visualized using 3,3'-diaminobenzidine (DAB) substrate (Dako) for 1 minute, and images were obtained under a light microscope.

RNA-seq Analysis

Total RNA or mRNA was isolated from tumor tissues or cultured cells for high-throughput RNA sequencing. Complementary DNA libraries were constructed and sequenced on the Illumina HiSeq platform by GENEWIZ. High-quality clean reads were mapped to the human reference genome GRCh38 with Bowtie 2, and gene expression levels were quantified and normalized as fragments per kilobase of exon per million mapped reads (FPKM). Significantly differentially expressed genes were visualized in a heatmap. Gene ontology enrichment analysis was performed using the DAVID web-based tool, and all statistical graphs and visualizations were generated using the R programming environment.

***In Vivo* Tumor Growth Assays**

Our study examined male and female animals, and similar findings are reported for both sexes. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Tianjin Medical University Cancer Institute and Hospital (Approval No. AE-2024017) and conducted in accordance with relevant guidelines. Six-week-old male BALB/c nude mice and SCID/Beige mice were purchased from GemPharmatech (Beijing, China). Cells were subcutaneously inoculated into the flank region of BALB/c nude mice at a density of 2×10^6 cells per mouse, with five animals per group. Mice were euthanized and tumors were dissected four weeks post-injection. Tumor volume was calculated using the formula: (length × width^2)/2.

CUT&Tag Assay

CUT&Tag assays were performed using the HyperActive Universal CUT&Tag Assay Kit for Illumina Pro (Vazyme, TD904) following the manufacturer's protocol. Concanavalin A-coated magnetic beads were used to immobilize 1×10^4 to 1×10^5 cells, which were then incubated with primary antibodies against the target protein and corresponding secondary antibodies. Protein A/G-Tn5 transposase fusion protein was

then added to cleave and tag DNA regions bound by the antibodies of interest. DNA fragments were purified, amplified via PCR, and sequenced on an Illumina sequencing platform. Raw sequencing reads were quality-trimmed, aligned to the reference genome using Bowtie2, and peak calling was performed using the MACS2 algorithm.

Statistical Analysis

Data processing and statistical evaluation were performed using SPSS 21.0 and GraphPad Prism 9 software. Quantitative results are expressed as the mean ± standard deviation (SD). Statistical comparisons were carried out using suitable analytical methods, including two-tailed Student's t-test, chi-square test, and one-way or two-way ANOVA, as specified in the corresponding figure legends. All experimental assays were performed independently in triplicate. A P-value < 0.05 was regarded as statistically significant.

DATA AVAILABILITY

The authors declare that the data are present in the paper and/or the supplementary information. Additional data supporting the present study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

FM and HZ conceived and supervised the project. YG provided administrative support. HZ, and FM performed the experiments and analyzed the data. YG and YR collected the tissue samples and contributed to the histological analysis. FM and HZ wrote the manuscript. All authors approved the final manuscript.

COMPET INGINTERESTS

The authors declare no conflict of interest.

CONSENT FOR PUBLICATION

All contributing authors agree to the publication of this article.

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