IncRNA DUXAP8 Facilitates Multiple Malignant Phenotypes and Resistance to PARP Inhibitor in HCC via Upregulating FOXM1

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In this study, we examined the clinical significance and molecular mechanisms of a long non-coding RNA (IncRNA), double homeobox A pseudogene 8 (DUXAP8) in hepatocellular carcinoma (HCC). DUXAP8 expression was compared using quantitative real-time PCR in HCC versus adjacent tissues and in HCC cell lines versus normal hepatic epithelial cells. The correlations between DUXAP8 level and clinicopathological features were analyzed. Assays including MTT, colony-forming analysis, Transwell assay, western blot, xenograft formation, experimental metastasis, luciferase assay, RNA pull-down, and RNA immunoprecipitation were used to examine DUXAP8-induced malignant phenotypes, its regulation on forkhead box protein M1 (FOXM1), and the importance of FOXM1 in mediating DUXAP8 phenotypes. Our results showed that DUXAP8 was significantly upregulated in HCC tissues or cell lines associated with tumors of advanced grades, tumors that were positive for lymph node metastasis, and patients with poor overall survival. DUXAP8 was essential in maintaining multiple malignant phenotypes (including resistance to olaparib) both in vitro and in vivo. Mechanistically, DUXAP8 upregulated FOXM1 expression by splicing miR-485-5p and interacting with the RNA-binding protein Fused in Sarcoma (FUS). Functionally, FOXM1 essentially mediated the oncogenic phenotypes of DUXAP8. Collectively, DUXAP8 acts through two distinct mechanisms to upregulate FOXM1 and becomes a pleiotropic oncogenic IncRNA in HCC.

INTRODUCTION
Hepatocellular carcinoma (HCC) is the predominant form of primary liver cancer, accounting for 70%–85% of all HCC cases worldwide.1 In China, the prevalence of chronic infection of hepatitis B virus and hepatitis C virus significantly contributes to the high morbidity (responsible for >55% of all HCC cases worldwide) and mortality of HCC.2 Although vaccination and other prevention strategies may lower the incidence and mortality of HCC by more than 40% through 2030 in China, it remains an aggressive malignancy with a median survival between approximately 6 and 20 months.3 The complex and yet not so well-understood pathogenesis suggest that a multimodality strategy is required for HCC therapy. Therefore, it is critical to identify new diagnostic and/or prognostic biomarkers and to understand molecular pathogenic mechanisms that may facilitate the development of effective therapeutic strategies.

The defect in the DNA repair system is a significant pathogenic factor in cancer development. Poly(ADP-ribose) polymerase (PARP) is a family of enzymes that catalyze the posttranslational conjugation of poly(ADP-ribose) to target proteins and regulate multiple cellular processes. The best characterized PARP family member, PARP1, plays an essential role in the base excision repair (BER) pathway of repairing DNA single-strand breaks.4 While the breast cancer gene 1 (BRCA1) and BRCA2 critically regulate the homologous recombination (HR) pathway repairing DNA double-strand breaks,5 in cells with deficient BRCA1 or BRCA2 functions, BER is the default DNA repair pathway. Therefore, targeting PARP signaling may lead to the death of cancer cells with mutations in BRCA1 and/or BRCA2, a phenomenon known as synthetic lethality.6 In support of this concept, a few PARP inhibitors (PARPis), including olaparib, rucaparib, and niraparib, have been approved by the Food and Drug Administration (FDA) and the European Medicine Agency for treatment of ovarian or breast cancers, particularly those bearing BRCA mutations (https://www.fda.gov). Meanwhile, more PARPis are being tested in pre-clinical and clinical trials. The success of the PARPi in cancer treatment is also accompanied by the increasing realization that many patients do develop resistance through diverse mechanisms.7 For example, Fang et al.7 recently reported that forkhead box protein M1 (FOXM1), a transcription factor and a master regulator of DNA damage repair, disrupted the sensitivity of cells to PARPis. For HCC, a few clinical trials on PARPis have been performed, and the available results are disappointing,8 which suggests the presence of mechanisms resistant to PARPis. Identifying these mechanisms will not only help to predict treatment response of HCC patients to PARPi but also promote the development of therapies that confer sensitivity to PARPis.

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Mounting evidence suggests that long noncoding RNAs (lncNRAs)—non-protein-coding RNAs with more than 200 nucleotides and capable of interacting with DNA, RNA, and proteins—play diverse roles in cancer progression and function not only as diagnostic biomarkers but also as therapeutic targets. Among them, the double homeobox A pseudogene 8 (DUXAP8) is a pseudogene-derived lncRNA upregulated in a variety of human cancers and serves as a pan-cancer diagnostic and/or prognostic biomarker. Earlier studies on HCC showed that DUXAP8 expression increased, was an unfavorable prognostic biomarker, and promoted the proliferation and invasion of tumor cells. However, the mechanisms underlying the oncogenic activities of DUXAP8 remain largely unknown. In the present study, we aim to characterize biological effects and molecular mechanisms of DUXAP8 in HCC. Through bioinformatic analysis, we identified that DUXAP8 may interact with miR-485-5p, which may also interact with FOXM1. This clue prompted us to focus on the DUXAP8/miR-485-5p/FOXM1 axis and its importance in HCC development.

RESULTS

DUXAP8 Was Upregulated in HCC Tissues and Cell Lines and Associated with Worse Prognosis

A recent study identified DUXAP8 as a robustly upregulated IncRNA that associated with poor overall survival of HCC patients. To explore the mechanisms by which DUXAP8 act in HCC, we collected 52 pairs of HCC tissues and matching para-tumor tissues to assess the expression of DUXAP8. As shown in Figure 1A, DUXAP8 was significantly upregulated in cancer tissues compared with para-tumor tissues (p < 0.001). Further stratification analyses revealed that DUXAP8 expression was markedly elevated in Tumor Node Metastasis (TNM) stage-III/IV tumors (n = 27), compared with that in stage-I/II tumors (n = 25; p < 0.01; Figure 1B), and also in those positive for lymph node metastasis (n = 32) compared with those negative (n = 20; p < 0.01; Figure 1C). Consistently, The Cancer Genome Atlas (TCGA) database analysis through the Gene Expression Profiling Interactive Analysis (GEPIA) website (http://gepia.cancer-pku.cn/index.html) showed that: first, the DUXAP8 level from HCC tissues (n = 369) was robustly higher than that from normal tissues (n = 160; p < 0.05; Figure 1D); second, a higher level of DUXAP8 (n = 182) was significantly correlated with worse overall survival (p = 0.0035; Figure 1E) of HCC patients; and third, there was no significant correlation between DUXAP8 level and the disease-free survival of HCC patients (p = 0.13; Figure 1F). When examining DUXAP8 expression among different HCC cell lines—namely, HCCC-9810, BEL-7402, Huh7, SMMC-7721, HepG2, and HepG2/ADR—we observed that its level was markedly higher than in normal liver epithelial cell line THLE-3 (all p < 0.05; Figure 1G). With all data taken together, DUXAP8 is upregulated in HCC tissues.
Figure 2. DUXAP8 Promoted Multiple Malignant Phenotypes of HCC Cells

(A) Huh7 and BEL-7402 cells were stably transfected with three distinct shDUXAP8 vectors. The expression of DUXAP8 was examined by quantitative real-time PCR and compared to that in cells transfected with shNC. (B and C) The cell viability of shNC versus shDUXAP8 Huh7 (B) and BEL-7402 (C) cells was examined by MTT assay. (D and E) Colony-forming assay was performed to assess the long-term proliferation of shNC versus shDUXAP8 Huh7 (D) and BEL-7402 (E) cells. Representative images of colonies formed are presented on the left, and the quantification of the number of colonies formed is presented on the right. (F and G) The migration and invasion of shNC versus shDUXAP8 Huh7 (F) and BEL-7402 (G) cells were examined by Transwell assays. Representative images of migrated cells

(legend continued on next page)
and cell lines, and its elevated expression correlates with tumors of higher malignancy or patients with worse prognosis, supporting that DUXAP8 is an oncogenic lncRNA in HCC.

DUXAP8 Promoted Multiple Malignant Phenotypes of HCC Cells
To understand the molecular mechanisms underlying the action of DUXAP8 in HCC, we chose BEL-7402 and Huh-7 cells as the model system, which represents HCC cells expressing high levels of endogenous DUXAP8, and adopted the loss-of-function strategy by stably expressing three different shRNA sequences specifically targeting DUXAP8 (shDUXAP8—#1, #2, and #3) in these cells. As shown in Figure 2A, shDUXAP8#1 demonstrated the most robust effect in knocking down DUXAP8 in both cells (both ps < 0.001, when compared to shNC [negative control]-expressing cells) and thus was used for subsequent experiments, where it was referred to as shDUXAP8. Corresponding to the reduction of DUXAP8, when compared to the corresponding shNC cells, shDUXAP8 cells presented significantly reduced viability (as determined by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide [MTT] assay; both ps < 0.001 [Figures 2B and 2C]), colony formation (as shown by colony-forming assay; p < 0.01 for Huh7 [Figure 2D] and p < 0.001 for BEL-7402 cells [Figure 2E]), migration and invasion (as measured using Transwell migration/invasion assay; p < 0.001 for migration and p < 0.01 for invasion [Figures 2F and 2G]), and epithelial-to-mesenchymal transition ([EMT] as represented by increased expression of epithelial biomarker E-cadherin and reduced expressions of mesenchymal biomarkers, N-cadherin, vimentin, and slug; all p < 0.05 [Figures 2H and 2I]), supporting that DUXAP8 plays an essential role in maintaining multiple malignant phenotypes of HCC cells.

DUXAP8 Stimulated Xenograft Growth and Experimental Metastasis of HCC Cells
In addition to examining the effects of DUXAP8 on HCC cells cultured in vitro, we established xenograft models using shDUXAP8 or shNC BEL-7402 cells and monitored the in vivo xenograft growth. Consistent with the viability-suppressing effect of shDUXAP8 in vitro, shDUXAP8 BEL-7402 cells generated smaller xenografts (Figure 3A) that resulted from significantly thwarted in vivo growth (p < 0.01, when compared to xenografts derived from shNC cells; Figure 3B). Consistently, staining for Ki67, a biomarker for proliferating cells, showed that the proliferating activity was markedly lower in shDUXAP8 xenografts than in shNC ones (Figure 3C). Furthermore, we compared the experimental lung metastasis of shDUXAP8 and shNC HCC cells. Figure 3D showed that knocking down DUXAP8 in BEL-7402 cells potently reduced the metastatic capacity of these cells. The difference in the number of metastatic nodules was **p < 0.01; ***p < 0.001.

(Left two panels) and invaded cells (middle two panels), as well as the quantification of the number of migrated or invaded cells per field (right panel) are shown. (H) The expressions of EMT-related biomarkers, including E-cadherin, N-cadherin, Vimentin, and Slug, in shNC versus shDUXAP8 Huh7 and BEL-7402 cells were examined by western blot. GAPDH was examined as the internal control. (I) The relative expressions of indicated genes were quantified from western blot. *p < 0.05; **p < 0.01; ***p < 0.001.
statistically significant (p < 0.001, when compared to shNC-expressing cells; Figure 3E) and evident from hematoxylin and eosin (H&E)-stained lung tissues. These data evidence the in vivo significance of DUXAP8 in promoting cancer progression and metastasis.

Knocking Down DUXAP8 Enhanced the Sensitivity of HCC Cells to PARPis

PARPis presented promising, yet not ideal, therapeutic benefits for HCC.10,16 To assess whether DUXAP8 impacts the sensitivity of
HCC cells to PARPi, we compared the half maximal inhibitory concentration (IC50) of olaparib, a PARPi approved by the FDA for cancer treatment, between shDUXAP8 and shNC (both Huh7 and BEL-7402 cells). By measuring the viability of HCC cells in response to different doses of olaparib, we calculated that the IC50 for shDUXAP8 Huh7 cells was $78.25 \mu M$, significantly lower than that for shNC Huh7 cells (IC50 = $187.33 \mu M$; p < 0.01; Figure 4A). Similarly, the IC50 for shDUXAP8 BEL-7402 cells was $101.39 \mu M$, also significantly lower than that for shNC BEL-7402 cells (IC50 = $157.69 \mu M$; p < 0.01; Figure 4B). Consistently, the strongest in vivo inhibitory effect on xenograft growth was observed when olaparib was used on shDUXAP8 xenografts (olaparib+shDUXAP8), followed by when olaparib was used on shNC xenografts (olaparib+shNC), as reflected by both the sizes of xenografts isolated on day 21 after the initial inoculation of different BEL-7402 cells (Figure 4C) and by the growth curve of xenografts (p < 0.001 and p < 0.01, when comparing the saline and olaparib +shDUXAP8 group with the olaparib +shNC group, respectively; Figure 4D). Further immunohistochemistry analysis on the xenografts showed that the expression of FOXM1 was most robustly reduced in the olaparib+shDUXAP8 xenografts (Figure 4E). In vitro, olaparib treatment induced, in a time-dependent manner, the expressions of DNA damage repair genes, including FOXM1, BRCA1, and RAD51, on both the protein (Figures 4F and 4G) and mRNA (Figures 4H and 4I) levels. Knocking down DUXAP8 was sufficient to suppress olaparib-induced upregulation of these genes (Figures 4F–4I). Similar effects were also observed on genes regulating cell cycle, including CDK6 and CCNB1 (Figures 4H and 4I), suggesting that DUXAP8 essentially controls the sensitivity of HCC cells to PARPi, which may work through its regulation on the expressions of multiple DNA repair and cell-cycle genes.

**DUXAP8 Sponged miR-485-5p and Released the Suppression of the Latter on FOXM1**

Since FOXM1 is an upstream regulator for other DNA repair genes, including FOXM1, BRCA1, and RAD51, we specifically focused on the relationship and regulation between DUXAP8 and FOXM1. Analysis of 374 HCC samples from TCGA database showed that the DUXAP8 level was positively correlated with that of FOXM1 (p < 0.001; Figure 5A). Quantitative real-time PCR analysis showed that FOXM1 was significantly upregulated in SMMC-7721, HepG2, BEL-7402, and Huh-7 cells, but not in HCCC9810 cells, when compared to normal THLE-3 cells (Figure 5B). Bioinformatic analysis showed that both DUXAP8 and FOXM1 may bind to miR-485-5p, where DUXAP8 contains two potential miR-485-5p-binding sequences (Figure 5C) and FOXM1 contains only one (Figure 5F). Luciferase analysis showed that miR-485-5p
mimic specifically inhibited the luciferase activity driven by the wild-type (WT) 1 and WT 2 sequences of DUXAP8 (p < 0.01, when compared to cells treated with mimic NC), and the inhibition was compromised when either or both sites were mutated (MUT) (Figure 5D). Correspondingly, we detected the failure of miR-485-5p mimic to inhibit the endogenous FOXM1 mRNA level in BEL-7402 cells transfected with the luciferase reporter construct containing the WT binding sequence, yet FOXM1 mRNA was significantly reduced in cells co-transfected with miR-485-5p mimic and the luciferase reporter construct containing mutant sequence for site 1, 2, or 1/2 (Figure 5E). Similarly, for the MUT FOXM1 binding sequence, the luciferase activity was no longer affected by miR-485-5p mimic (Figure 5G), suggesting that DUXAP8 sponges miR-485-5p and releases the suppression of the latter on FOXM1.

**DUXAP8 Stabilized FOXM1 mRNA by Binding to FUS**

In addition to sponging microRNA (miRNA), we also examined the potential involvement of FUS, an mRNA stabilizer, in upregulating FOXM1 by DUXAP8. An RNA immunoprecipitation (RIP) assay showed that, in both Huh7 and BEL-7402 cells, FUS bound to both DUXAP8 and FOXM1 mRNA (p < 0.001, when compared to immunoglobulin G [IgG]; Figures 6A and 6B). The interaction between DUXAP8 and FOXM1 was further confirmed using an RNA pull-down assay (Figures 6C and 6D). To test whether DUXAP8, by acting through FUS, stabilizes FOXM1 mRNA, we measured the decay of FOXM1 mRNA in shNC versus shDUXAP8 Huh7 and BEL-7402 cells in the absence or presence of FUS overexpression. As shown in Figures 6E and 6F, knocking down DUXAP8 (shDUXAP8) specifically accelerated the decay of FOXM1 mRNA in both cells (p < 0.001, when compared to shNC cells), whereas overexpressing FUS in shDUXAP8 cells (shDUXAP8 + FUS) rescued the decay-stimulating effect of shDUXAP8 (p < 0.01; comparing shDUXAP8 with shDUXAP8+FUS cells).

**miR-485-5p Inhibited Multiple Malignant Phenotypes and PPARi Resistance of HCC Cells**

Since DUXAP8 plays an essential role in maintaining multiple malignant phenotypes as well as the sensitivity of HCC cells to the PARPi olaparib, and since we showed earlier that DUXAP8 sponges miR-485-5p and thus upregulates FOXM1, it is therefore interesting to assess the role of miR-485-5p in regulating the malignancy of HCC. For this purpose, we transfected both Huh7 and BEL-7402 cells with miR-485-5p mimic, which significantly boosted the miR-485-5p level, when compared to non-transfected (control) or control-mimic (mimic-NC)-transfected cells (p < 0.001; Figure 7A). In response to the elevated level of miR-485-5p, we noticed that the viability (Figures 7B and 7C), long-term proliferation (Figures 7D and 7E), migration/invasion (Figures 7F and 7G), FOXM1 expression, and EMT (Figures 7H and 7I) were all significantly and potently inhibited, whereas the sensitivity to olaparib was significantly enhanced (IC50 was reduced from 200.41 μM in mimic-NC-treated Huh7 cells to 130.01 μM in miR-485-5p mimic-treated Huh7 cells and from 206.31 μM in NC-treated BEL-7402 cells to 105.12 μM in miR-485-5p mimic-treated BEL-7402 cells; Figures 7J and 7K), suggesting that miR-485-5p is a tumor-suppressive miRNA that inhibits multiple malignant phenotypes of HCC cells.

**FOXM1 Essentially Mediated the Oncogenic Activities of DUXAP8**

Next, we examined the significance of FOXM1 on the oncogenic activities of DUXAP8 by overexpressing FOXM1 in shDUXAP8 Huh7...
and BEL-7402 cells (shDUXAP8+FOXM1). Consistent with earlier findings, the expression of FOXM1 was significantly reduced in shDUXAP8 cells alone (shDUXAP8+vector) (p < 0.05, when compared to the NC cells (shNC+vector)) but was resumed in shDUXAP8+FOXM1 cells (Figure 8A). Correspondingly, the shDUXAP8-induced reductions of cell viability (Figures 8B and 8C) and migration/invasion (Figures 8D and 8E), or increased sensitivity to olaparib (Figures 8F and 8G) (all p < 0.01, when compared to shNC+vector cells), were all reversed by overexpressing FOXM1 in these cells. Combined with the earlier finding that DUXAP1 upregulated the expression of FOXM1, these data suggest that FOXM1 plays a critical role in mediating the oncogenic activities of DUXAP8.

**DISCUSSION**

Pseudogenes are generated when natural mutations accumulate on a protein-coding gene, leading to the decay or degradation of the transcript.17 The sequence similarity between pseudogenes and the corresponding protein-coding genes confers the potential for the former to regulate cellular processes in a sequence-specific pattern.16 Mounting evidence suggests not only that most pseudogenes are actively transcribed, but also that lncRNAs transcribed from pseudogene loci may modulate gene transcription and epigenetic state and function as guides, tethering molecules, or competitive endogenous RNAs (ceRNAs) to sponge miRNAs.19 In this study, we focused on the pseudogene-derived lncRNA DUXAP8 and presented substantial evidence that DUXAP8 is an oncogenic lncRNA during HCC development: it was essential for maintaining a repertoire of malignant phenotypes of in vitro cultured HCC cells, including cell viability, long-term proliferation, migration/invasion, and EMT; knocking down DUXAP8 suppressed the xenograft growth and HCC metastasis in vivo; from the clinical perspective, DUXAP8 expression was upregulated in HCC tissues; and its level correlated with that of tu-

![Figure 7. miR-485-5p Inhibited Multiple Malignant Phenotypes of HCC Cells](image)

![Figure 7.](image)

 previous studies also reported the tumor-suppressive activities of miR-485-5p in human cancers, including gastric cancer,26,27 NSCLC,28 breast cancer,29,30 and thyroid cancer,31,32 and in HCC.33–36 In HCC, distinct lncRNAs all sponge miR-485-5p and up-regulate different downstream targets to accomplish their oncogenic activities.33,35,36 These studies, when combined with the present one, support the significance of miR-485-5p as a signaling hub linking myriad lncRNAs with downstream targets to deliver malignant phenotypes. Consistently, when it was overexpressed in two different HCC cell lines, we showed that miR-485-5p significantly suppressed multiple malignant phenotypes. It would be interesting for future studies to comprehensively analyze the signaling wheel that hubs miR-485-5p and assess the therapeutic potential/safety of boosting its expression in cancer.

FUS is an RNA-binding protein that regulates pre-mRNA splicing and mRNA transport, stability, and translation.17 The dreamBase analysis suggests that FUS protein contains interaction sites for 5,106 distinct pseudogenes,20 yet the specific interaction between FUS and DUXAP8...
Figure 8. FOXM1 Essentially Mediated the Oncogenic Activities of DUXAP8

shNC versus shDUXAP8 Huh7 and BEL-7402 cells were transiently transfected with FOXM1-expressing vector or empty vector (NC). (A) The expression level of FOXM1 was detected by western blot with representative image shown on the left and the quantification on the right. β-actin was detected as the loading control. (B and C) The cell viability of indicated Huh7 (B) and BEL-7402 (C) cells was examined by MTT assay. (D and E) The migration and invasion of indicated Huh7 (D) and BEL-7402 (E) cells were examined by Transwell assays. Representative images of migrated cells (left upper panels) and invaded cells (left lower panels), as well as the quantification of the number of migrated or invaded cells per field (right panel) are shown. (F and G) Indicated Huh7 (F) and BEL-7402 (G) cells were treated with olaparib at different concentrations. The logIC50 was calculated. *p < 0.05; **p < 0.01; ***p < 0.001.
or the biological significance of this crosstalk has not been reported before. Here, using RIP and RNA pull-down assays, we showed for the first time that these two molecules interacted. Functionally, knocking down DUXAP8 promoted the decay of FOXM1 mRNA, which was stabilized by overexpressing FUS, supporting the oncogenic activities of FUS. The functional significance of FUS in HCC, however, is under debate, with some studies supporting the anti-cancer activities while the others report pro-tumor activities. Guo et al. reported that lncRNA SNHG6 was an oncogene for HCC and that one mechanism underlying the oncogenic activities of SNHG6 was by sponging miR-1297 and releasing its inhibition on FUS, the latter impeding the export of MAT1A mRNA from nucleus, downregulating MAT1A protein synthesis, and contributing to genome-wide hypomethylation. In addition, analysis of the HCC gene expression profile GEO: GSE49515 identified FUS as a hub gene whose expression correlated with worse overall survival of HCC patients, which is also consistent with our findings from TCGA database analysis, which revealed that FUS was upregulated in HCC. Therefore, future studies should focus on characterizing the functional roles of FUS in HCC, either as an oncogene or as a tumor suppressor, and understand the molecular mechanisms mediating such functional roles.

FOXM1 is a transcription factor presenting pleiotropic oncogenic activities in human cancers, ranging from proliferation/apoptosis to migration, to EMT, angiogenesis, metastasis, and drug resistance. The expression level of FOXM1 in cancer is regulated on multiple levels, including the transcriptional regulation by distinct transcription factors; post-transcriptional regulation by miRNAs; and post-translational modifications such as phosphorylation, acetylation, methylation, ubiquitination, and sumoylation. In this study, we revealed a novel signaling cascade controlling FOXM1 expression in HCC, the DUXAP8/miR-485-5p/FOXM1 axis. We also showed that DUXAP8 may interact with FUS to stabilize FOXM1 mRNA. Functionally, the upregulated FOXM1 was a critical driver in mediating the oncogenic potential of DUXAP8 and was sufficient to induce the proliferation and the migration of HCC cells, as well as their resistance to PARPis. In support of our findings, Fang et al. reported that olaparib induced the expression and nuclear localization of FOXM1, which, in turn, upregulated other HR repair genes such as BRCA1 and RAD51, and contributed to olaparib-induced adaptive responses. Besides FOXM1, other factors such as epidermal growth factor receptor (EGFR) and c-MET heterodimer, also regulate resistance to PARPis. Therefore, it would be interesting for future studies to examine whether DUXAP8 acts through other mechanisms to regulate HCC resistance to PARPis.

In summary, here, we report the biological and clinical significance of the DUXAP8/FOXM1 axis in HCC and reveal two novel regulatory mechanisms between these two molecules. This study provides substantial evidence that targeting DUXAP8 or upregulating miR-485-5p suppresses HCC progression and enhances the sensitivity to PARPis. Considering the extended regulatory networks known to involve DUXAP8, miR-485-5p, or FOXM1 in cancer development, it is important to assess the importance of this axis in other types of cancer.

MATERIALS AND METHODS

Human Samples and Database Analysis
A cohort of 52 pairs of tumor tissues and matching para-tumor normal tissues were obtained during surgery between January 2018 and October 2019 from HCC patients admitted into the Department of General Surgery, Xiangya Hospital, Central South University (Hunan, P.R. China). The diagnosis was established by pathological examinations. Clinicopathological features of all HCC cases were recorded and are summarized in Table 1. Written consents were obtained from all participants, and the study was approved by the Ethics Committee of Xiangya Hospital. TCGA analysis was performed using the GEPIA as described previously.

Human Cancer Cell Lines and Reagents
The normal human liver epithelial cell line THLE-3; HCC cell lines HCCO9810, BEL-7402, HUH-7, SMMC-7721, and HepG2; and the
multiple drug-resistant HepG2/adriamycin (HepG2/ADR)\textsuperscript{32} were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, P.R. China). THLE-3 cells were cultured in BEGM medium supplemented with the BEGM Bullet Kit (Lonza, Walkersville, MD, USA), and all HCC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. All cells were maintained in a sterile humidified atmosphere containing 5% CO\textsubscript{2} at 37°C.

Olaparib (AZD2281) was purchased from MCE (Shanghai, P.R. China), dissolved in DMSO, and reconstituted to 10 mM with H\textsubscript{2}O.

**Reverse Transcription Followed by Quantitative Real-Time PCR**

Quantitative real-time PCR was performed as described previously\textsuperscript{52} using the following primers for the corresponding human genes: U6 (internal control for lncRNA or miRNA) forward primer, 5'-CTTCTGGACGACA-3', and reverse primer, 5'-AACGCCCTCAGAA TTGGGT-3'; DUXAP8 forward primer, 5'-AGGATGGAGTCT CGGTATATGC-3', and reverse primer, 5'-GGAGTTTGTCTTCTT CTTTTTT-3'; miR-485-5p forward primer, 5'-AGAGGCTGGCGGT GAT-3', and reverse primer 5'-GAAACGTCTGCTGTACCTC-3'; FOXM1 forward primer, 5'-CTTGGCAATGCAAGGTCTCCT-3', and reverse primer, 5'-CTTGGATTGCGGTCTTCTGCTG-3'; CDK6 forward primer, 5'-GATAAAGTTCAAGAGCCCCTGAAG-3', and reverse primer, 5'-GGATGCTACTCTGCTGTGA-3'; CCNB1 forward primer, 5'-GACCTTGTCAGCTTTTCTCCTG-3', and reverse primer, 5'-GGTATTTGGTCTGACTCTGGTC-3'; BRCAl forward primer, 5'-CTGAAGACTGTCAGGGCTAC-3', and reverse primer, 5'-GGGTCAGTTGAGGCTGCTG-3'; RAD51 forward primer, 5'-TCTCTGGACAGATGTTCTGGA-3', and reverse primer, 5'-TAAAGGGCGGTGGCACTGTCTA-3'; GAPDH (internal control for mRNA) forward primer, 5'-GTCCTCTCTGAGTTCAACAGGG-3', and reverse primer, 5'-ACCACCCCTGTGC TGTAGCCCA-3'. The relative expression of a target gene was calculated using the 2\textsuperscript{ΔΔCt} method.\textsuperscript{53}

**Construction of Lentivirus and Generation of Stable Cells**

shDUXAP8, shNC, human miR-485-5p mimic, and mimic NC were purchased from GenePharma (Shanghai, P.R. China). shDUXAP8 or shNC sequences were cloned into lentiviral vector pLKO.1-puro purchased from GenePharma (Shanghai, P.R. China). shDUXAP8, shNC, human miR-485-5p mimic, and mimic NC were transduced into target cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

**Cell Viability Assay**

Cell viability was measured using the MTT assay. Briefly, cells were seeded in 96-well plates at a density of 2 \times 10\textsuperscript{5} cells per milliliter and incubated for 6, 12, 24, 48, or 72 h. MTT solution (5 mg/mL in PBS, 20 mL per well; Sigma) was added to each well and allowed to incubate with cells at 37°C for 3 h. The medium was then replaced with 100 mL dimethyl sulfoxide (DMSO; Sigma) and incubated with cells for a further 2 h in the dark at room temperature. Finally, the optical density of formazan crystals dissolved by DMSO (proportional to the number of live cells) was measured with the Bio-Rad 550 Microplate Reader at 490 nm.

**Coloncy-Forming Assay**

The long-term proliferation of cells was measured using a colony-forming assay. Briefly, target cells were seeded into a 24-well plate at 200 cells per well. After incubating the cells at 37°C for 10 days, cells were fixed with 100% methanol (Sigma) at room temperature for 10 min and then stained with 0.5% crystal violet (Sigma) at room temperature for 5 min. The number of cell colonies was counted under a light microscope.

**Transwell Migration/Invasion Assay**

A Transwell assay was performed as previously described.\textsuperscript{54} Briefly, the single-cell suspension of target cells was seeded into the top well (1 \times 10\textsuperscript{5} cells per well) of Transwell inserts (8.0 μm; Corning, Lowell, MA, USA) overnight. After incubating the cells at 37°C for 10 h, 200 μL DMEM containing 10% fetal bovine serum was added. After 24 h, the non-migrating/invasive cells were removed from the top of the membrane, and those that migrated or invaded through the membrane were fixed in 95% methanol, stained with 1% crystal violet, and counted under an inverted microscope (100×).

**Western Immunoblots**

Total proteins were extracted from cultured cells using RIPA buffer (Thermo Fisher Scientific) and separated on 10% SDS-PAGE gel. Following the transfer of separated proteins onto a polyvinylidene difluoride membrane, the membrane was blocked with 5% nonfat milk in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.5% Tween 20) at room temperature for 1 h, washed three times in TBST, and incubated with one of the following primary antibodies (all from Cell Signaling Technology, Danvers, MA, USA, unless otherwise indicated): E-cadherin (1:1,000; #14472), N-cadherin (1:1,000; #13116), Vimentin (1:1,000; #5741), Slug (1:1,000; #9858), FOXM1 (1:1,000; #5436), BRCAl (1:1,000; #14823), RAD51 (1:1,000; #8875), or β-actin (1:5,000; #3700; internal control) at 4°C overnight. After three washes with TBST, the membrane was incubated with horseradish-peroxidase-conjugated secondary antibodies at room temperature for 2 h.
The signal was developed using an enhanced chemiluminescence system (Beyotime, Jiangsu, P.R. China) according to the manufacturer’s instructions.

In Vivo Xenograft Growth and Experimental Metastasis Assays
Female athymic BALB/c nude mice (4–6 weeks old) were purchased from Hunan SJA Laboratory Animals (Hunan, P.R. China) and maintained under pathogen-free conditions. To monitor in vivo xenograft growth, BEL-7402 cells stably transfected with shDUXAP8 versus shNC were subcutaneously injected into the dorsal flank region of each mouse (1 × 10^6 cells per injection; n = 4 per group). Tumor growth was measured every 3 days using a vernier caliper, and xenografts were examined. To examine the experimental metastasis, BEL-7402 cells stably transfected with shDUXAP8 versus shNC were intravenously injected into each mouse through the tail vein (1 × 10^6 cells per injection; n = 5 per group). After 30 days, mice were sacrificed, and the lungs were excised, imaged, and processed for histological analysis. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Xiangya Hospital.

Histological Analysis
Upon fixation in 10% formalin and embedded in paraffin, mouse tissues were processed into 4-μm sections. Staining with H&E was performed using an H&E staining kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. For immunohistochemical (IHC) staining for Ki67 and FOXM1, tissue sections underwent antigen retrieval in boiling 10 mM citrate buffer (pH 6.0) for 10 min and blocked for endogenous peroxidase activity using 0.3% H2O2 for 10 min followed by non-specific binding using 5% normal goat serum for 1 h at room temperature. The tissue sections were incubated with anti-Ki67 (1:400, #9449) or anti-FOXM1 (1:600, #20459) antibody (both from Cell Signaling Technology) at 4°C overnight, followed by biotinylated secondary antibody at room temperature for 30 min, and then with Vectastain ABC-HRP solution (Vector Labs) at room temperature for 30 min, with TBST washes in between. The signal was developed using diaminobenzidine (DAB) substrate (Vector Labs), and the slides were counterstained with hematoxylin.

Luciferase Reporter Assay
We used the starBase platform (http://starbase.sysu.edu.cn/) and identified miR-485-5p as a miRNA that could interact with both DUXAP8 and FOXM1. Mutations were introduced into the potential binding sequences of DUXAP8 and FOXM1, respectively. The WT and MUT binding sequences were cloned into pRL-CMV luciferase reporter plasmid. BEL-7402 cells were co-transfected with the luciferase reporter plasmid and miR-485-5p mimic or control mimic (mimic NC) using Lipofectamine 3000. At 48 h following the transfection, luciferase activity was detected using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

RIP
To examine the potential binding between FUS and DUXAP8 or FOXM1 mRNA, a RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17-700, Millipore) following the manufacturer’s instructions. Briefly, whole-cell lysate was incubated with anti-FUS antibody (ab70381, Abcam, Cambridge, MA, USA) conjugated to protein A/G Sepharose beads at 4°C for 6 h. Immunoprecipitated RNA was then detected by qPCR and presented as a fold change relative to the amount of RNA co-precipitated with control IgG.

RNA Pull-Down Assays
WT and MUT DUXAP8 RNAs were in vitro transcribed using T7 RNA polymerase (Roche, Basel Switzerland), treated with RNase-free DNase I (Roche, Indianapolis, IN, USA), and purified with the RNeasy Mini Kit (QiAGEN, Germantown, MD, USA). Transcribed RNAs were biotin-labeled with the Biotin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN, USA). Biotinylated RNAs were then incubated with BEL-7402 cell lysates. Streptavidin-conjugated magnetic beads were added, and the eluted proteins were detected by western blot analysis.

Assay for mRNA Decay
The measurement of mRNA decay was performed as described previously. Briefly, shDUXAP8- or shNC-expressing BEL-7402 cells were transfected with either FUS-expressing vector or empty vector. After 48 h, cells were treated with actinomycin D to inhibit transcription (0 h). The FOXM1 mRNA level was then measured using quantitative real-time PCR at indicated time points and presented as a relative ratio to that at the 0-h time point.

Statistical Analysis
All data were analyzed by GraphPad Prism 6.0 and presented as means ± SD from at least three independent experiments (for in vitro assays) or from multiple mice within each group (for in vivo assays). Differences between experimental groups were assessed by the Student’s t test or one-way ANOVA. A p value of less than 0.05 was considered statistically significant.

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AUTHOR CONTRIBUTIONS
Guarantor of integrity of the entire study: Q.Z.; study concepts: Q.Z., Y.H., and H.-Y.Z.; study design: Q.Z., Y.H., and H.-Y.Z.; definition of intellectual content: Y.H. and Q.Z.; literature research: Y.H., L.X., and W.J.; clinical studies: Q.Z., Y.H., and L.X.; experimental studies: Y.H., Q.Z., L.X., and W.J.; data acquisition: X.Z. and Q.Z.; data analysis: X.Z. and Q.Z.; statistical analysis: X.Z. and Y.H.; manuscript preparation: Y.H. and Q.Z.; manuscript editing: Y.H. and Q.Z.; manuscript review: Y.H., Q.Z., and H.-Y.Z.

DECLARATION OF INTEREST
The authors declare no competing interests.
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