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HOXC11-mediated regulation of mitochondrial function modulates chemoresistance in colorectal cancer

Shicheng Chu[1†], Xiang Ren[1†], Lianmeng Cao[2], Chong Ma[1*] and Kai Wang[1*]

Abstract

Background Chemoresistance remains a significant challenge in colorectal cancer (CRC) treatment, necessitating a deeper understanding of its underlying mechanisms. HOXC11 has emerged as a potential regulator in various cancers, but its role in CRC chemoresistance remains unclear.

Methods Sulforhodamine B assay was employed to assess the cell viability of CRC cells following treatment with chemotherapeutic drugs. Immunofluorescence staining was performed to examine the subcellular localization of HOXC11 in normal and chemoresistant CRC cells. The Seahorse mito stress test was conducted to evaluate the mitochondrial respiratory function of CRC cells. Real-time PCR was utilized to measure the expression level and copy number of mitochondrial DNA (mtDNA).

Results Our findings revealed that HOXC11 was overexpressed in CRC cells compared to normal colorectal cells and correlated with poorer prognosis in CRC patients. Knockout of HOXC11 reversed acquired chemoresistance in CRC cells. Furthermore, we observed a functional subset of HOXC11 localized to the mitochondria in chemoresistant CRC cells, which regulated mitochondrial function by modulating mtDNA transcription, thereby affecting chemoresistance.

Conclusions In summary, our study reveals that HOXC11 regulates mitochondrial function through the modulation of mtDNA transcription, impacting chemoresistance in colorectal cancer cells. These findings underscore the significance of understanding the molecular mechanisms underlying chemoresistance and highlight the potential therapeutic implications of targeting mitochondrial function in CRC treatment.

Keywords Colorectal cancer, Mitochondrial DNA, Mitochondrial respiration, Chemoresistance, Chemotherapy

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Introduction

Chemotherapy plays a pivotal role in the treatment of colorectal cancer (CRC), significantly impacting patient outcomes and survival rates [1]. Common chemotherapeutic agents used in CRC treatment include 5-fluorouracil (5FU), irinotecan, and oxaliplatin [2, 3]. While chemotherapy has demonstrated efficacy in CRC management, the emergence of drug resistance poses a significant challenge [4–6]. Chemoresistance remains a formidable barrier to effective CRC treatment, undermining therapeutic efficacy and contributing to disease progression. Consequently, unraveling the mechanisms underlying chemoresistance in CRC is crucial for improving treatment outcomes and enhancing patient survival.

In addition to its role as the cell's powerhouse, mitochondria possess their own genetic system, known as mitochondrial DNA (mtDNA) [7]. Genes encoded on mtDNA primarily govern critical functions related to energy production and cellular metabolism. The regulation of mitochondrial gene expression plays a pivotal role in maintaining mitochondrial function and cellular homeostasis [8]. Mitochondrial gene transcription in mammals is orchestrated by a unique mitochondrial DNA-dependent RNA polymerase (POLRMT), progressing through three fundamental phases: initiation, elongation, and termination [9, 10]. The initiation step at promoters necessitates two crucial transcription factors: mitochondrial transcription factor A (TFAM) and B2 (TFB2M). This trio constitutes the essential mitochondrial core transcription machinery, indispensable for commencing transcription from mitochondrial DNA fragments [8, 9]. Numerous studies have underscored the significance of mitochondrial respiration in regulating cancer chemoresistance, including in CRC cells' resistance to chemotherapeutic agents [11–13]. However, the key regulators of mitochondrial respiration in CRC have yet to be fully elucidated. Therefore, further investigation into the factors influencing mitochondrial function in CRC cells holds significant promise in addressing chemoresistance in CRC.

HOXC11, identified as a transcription factor regulating gene transcription, has emerged as a significant player in various cancers, with its overexpression correlating with poorer overall survival and potentially influencing treatment outcomes [14–18]. In CRC, HOXC11 overexpression has been linked to poor prognosis and increased proliferation and invasion of cancer cells [19, 20]. However, its specific role in CRC chemoresistance remains unclear, and whether HOXC11 regulates mitochondrial function in CRC has not been reported.

Therefore, we aim to investigate whether HOXC11 plays a regulatory role in the chemoresistance of CRC. Our research reveals that HOXC11 is significantly overexpressed in CRC cells compared to normal colonic cells, and this elevated expression correlates significantly with poorer prognosis in colorectal cancer patients. Knocking out HOXC11 effectively reverses acquired chemoresistance. Further mechanistic studies uncovered a functional subset of HOXC11 localized within the mitochondria of chemoresistant CRC cells. This mitochondrial subpopulation of HOXC11 modulates mitochondrial respiration by regulating mtDNA transcription, thereby mediating chemoresistance in CRC cells. Overall, our findings shed light on the mechanistic link between HOXC11, mitochondrial function, and CRC chemoresistance, providing insights for future therapeutic strategies targeting this pathway.

Materials and methods

Cell culture and gene editing

Colorectal cancer cell lines HCT116 and HT29 were procured from a commercial source, the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a 5% CO2 atmosphere. Prior to experiments, routine testing was performed to ensure the absence of mycoplasma contamination.

The CRISPR/Cas9 system was employed to generate HOXC11 knockout cell lines. Guide RNAs (gRNAs) targeting exon 1 of the HOXC11 gene were individually cloned into the pSpCas9(BB)-2 A-Puro (PX459) V2.0 vector (Addgene). The sequences of the gRNAs used were as follows: gRNA#1: CTACTCCTCCTGCTATGCGG; gRNA#2: GCGCCCCTCTCCTTGCGCGA. HCT116 and HT29 cells were co-transfected with the PX459 plasmids harboring these gRNAs using Lipofectamine™ 3000 transfection reagent. After 48 h, transfected cells were subjected to puromycin selection (2 μg/mL), and puromycin-resistant colonies were isolated for clonal expansion. Successful knockout of HOXC11 was validated by Western blotting using a HOXC11-specific antibody.

Establishing chemoresistant cell lines

A gradual dose escalation strategy involving long-term exposure to increasing drug concentrations was implemented to generate 5FU, irinotecan, and oxaliplatin-resistant HCT116 and HT29 cell lines. The initial drug concentrations were 1 μM for 5-FU and irinotecan, and 0.2 μM for oxaliplatin. These concentrations were incrementally increased every two weeks until reaching final induced concentrations of 5 μM for 5-FU and irinotecan, and 4 μM for oxaliplatin, with the entire process spanning approximately 8 months.

Generation of signal peptide-fused HOXC11 expression constructs

Standard molecular cloning techniques were employed to fuse the nuclear localization signal (NLS) sequence (PKKKRKV) [21, 22], nuclear export signal (NES) sequence (MNLVDLQKKLEELELDEQQ) [23, 24], and mitochondrial targeting sequence (MTS) (MAASPHTLSSRLLTGCVGGSVWYLERRT) [25, 26] to the N-terminus of HOXC11. Additionally, a 6xHis tag was incorporated at the C-terminus of the HOXC11. The resulting fusion constructs (NLS-HOXC11-6xHis, NES-HOXC11-6xHis, and MTS-HOXC11-6xHis) were cloned into the pLenti6/V5-DEST™ Gateway™ Vector (Addgene) using Gateway™ recombination technology, following the manufacturer's instructions.

Lentiviral transduction

The constructed pLenti6/V5-DEST™ vectors harboring NLS-HOXC11-6xHis, NES-HOXC11-6xHis, and MTS-HOXC11-6xHis were co-transfected with packaging plasmids into HEK293T cells using a calcium phosphate transfection method. After 72 h, lentiviral supernatants were collected, filtered, and concentrated by ultracentrifugation. HOXC11 knockout HCT116 and HT29 cells were seeded in culture plates and transduced with the concentrated lentiviral particles carrying the respective constructs at 50–70% confluence. Polybrene (8 μg/mL) was included to enhance transduction efficiency. Twenty-four hours post-transduction, the medium was replaced with fresh growth medium, and after an additional 24 h, blasticidin selection (10 μg/mL) was initiated. Transduced cells were subsequently maintained in culture medium supplemented with 2 μg/mL blasticidin.

Data mining from TCGA database

The TCGA-COAD dataset (updated on May 29, 2020) was employed, encompassing 41 normal samples and 480 CRC samples. For the differential expression analysis of HOXC11, raw counts data were downloaded and preprocessed, with expression normalized using the DESeq2 package in R. The statistical significance of expression differences between normal and CRC tissues was assessed by Student's t-test, and box plots were generated with the ggplot2 package to visualize the results. For the Kaplan-Meier survival analysis, CRC patients were stratified based on chemotherapy treatments: 124 patients treated with 5-fluorouracil (5FU), 27 with irinotecan (Iri), 108 with oxaliplatin (Oxa), and 232 without chemotherapy based on clinical information provided in the TCGA dataset. Overall survival time was calculated from the date of diagnosis to the date of death or last follow-up. Patients were stratified into high and low HOXC11 expression groups using the median expression value as the cut-off. Survival analysis was then conducted using the Kaplan-Meier method with log-rank test in the 'survival' package, and survival curves were visualized using the 'survminer' package. All analyses were performed using R software.

Gene expression analysis by real-time PCR

Total RNA was isolated from CRC cells using a commercially available RNA extraction kit (QIAGEN) following the manufacturer's protocol. After DNase I treatment to remove genomic DNA contamination, complementary DNA (cDNA) was synthesized from the purified RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Real-time PCR was performed to quantify the expression levels of target genes using gene-specific primers and SYBR Green master mix (Applied Biosystems). The PCR amplification protocol included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at the appropriate temperature for 30 s, and extension at 72 °C for 30 s. The comparative Ct method was employed to analyze the expression levels of the target genes. The primer sequences used in these analyses are provided in Supplementary Table S1.

Protein analysis by Western blotting

To extract proteins, CRC cells were lysed using RIPA buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail. After quantifying protein concentrations, equal amounts of protein samples were loaded onto SDS-polyacrylamide gels for electrophoretic separation. The resolved proteins were then transferred onto PVDF membranes, which were subsequently blocked with a blocking buffer (5% non-fat milk in TBS-T) for 1 h at room temperature to prevent non-specific antibody binding. The membranes were incubated with primary antibodies targeting the target proteins overnight at 4 °C. Following washing steps, the membranes were probed with species-specific secondary antibodies conjugated to horseradish peroxidase. Finally, the protein bands were visualized using an enhanced chemiluminescence substrate detection system. The following antibodies were utilized: anti-HOXC11 antibody (Thermo Fisher Scientific, catalog #: TA502570, dilution: 1:1000); anti-α-Tubulin antibody (Invitrogen, catalog #: A11126, dilution: 1:5000). The membranes were cut prior to antibody hybridization to ensure that HOXC11 and the internal control, α-tubulin signal originated from the same loading. All replicates of Western blot experiments are provided in the Supplementary file.

Sulforhodamine B assay

Cells were seeded into 96-well plates at a density of 3000 cells per well and allowed to adhere for overnight

incubation. The following day, cells were exposed to varying concentrations of chemotherapeutic drugs for 72 h. After the treatment period, cells were fixed with ice-cold trichloroacetic acid (TCA) to precipitate and preserve cellular proteins. The fixed cells were then stained with a 0.4% sulforhodamine B (SRB) solution for 30 min at room temperature, followed by washing with 1% acetic acid to remove unbound dye. The SRB dye bound to cellular proteins was subsequently solubilized in a Tris base solution, and the absorbance was measured at 560 nm using an Infinite 200 PRO microplate reader (Tecan). Cell viability was determined by comparing the absorbance values of drug-treated cells to those of untreated control cells. Dose-response curves were generated by plotting the percentage of cell viability against the logarithm of drug concentration, and the data were fitted using a sigmoidal dose-response model.

Immunofluorescence analysis

Cells were seeded onto glass coverslips and allowed to adhere overnight. The following day, mitochondria were labeled by incubating the cells with 50 nM MitoTracker™ Red CMXRos (Invitrogen) in culture medium containing a reduced FBS concentration (1%) at 37 °C for 30 min. After mitochondrial staining, cells were washed with PBS, fixed with 4% formaldehyde, and permeabilized with 0.1% Triton X-100. Subsequently, cells were blocked with 5% FBS in PBST for 1 h at room temperature prevent non-specific antibody binding. To analyze the subcellular localization of HOXC11, cells were incubated with primary antibodies against HOXC11 overnight at 4 °C, followed by incubation with fluorescently labeled secondary antibodies for 1 h at room temperature. Additionally, cell nuclei were counterstained with DAPI for 20 min at room temperature. The coverslips were then mounted onto glass slides using Permount™ Mounting Medium, and fluorescence images were acquired using a Zeiss LSM 510 confocal laser scanning microscope.

Mitochondrial DNA content analysis

To assess the mitochondrial DNA (mtDNA) content in CRC cells, total genomic DNA was first isolated using a commercially available genomic DNA extraction kit (QIAGEN), following the manufacturer's instructions. Subsequently, real-time quantitative PCR was employed to measure the copy numbers of both mtDNA and nuclear DNA separately [27, 28]. Oligonucleotide primers were specifically designed to amplify a region of the mitochondrial genome (forward: ACACTTGGGGGTA GCTAAAGT; reverse: GATAGACCTGTGATCCATCG TG) and the nuclear-encoded 18 S ribosomal RNA gene (forward: CTCAACACGGGAAACCTCAC; reverse: C GCTCCACCAACTAAGAACG). The relative mtDNA copy number was then determined by comparing the cycle threshold (Ct) values obtained for the mitochondrial amplicon to those of the nuclear reference gene using the 2^-ΔΔCt method.

Analyzing protein-mtDNA interactions via immunoprecipitation

To investigate the interactions between specific proteins and mtDNA, an immunoprecipitation-based assay (mtDIP) was employed, adapting a previously described protocol [29–31]. Mitochondria were first isolated from CRC cells using a commercial mitochondrial isolation kit, followed by crosslinking of protein-DNA complexes with 1% formaldehyde treatment at room temperature for 10 min. The crosslinked mtDNA was then extracted and sheared into smaller fragments via sonication. These fragmented mtDNA molecules were incubated overnight at 4 °C with antibodies targeting HOXC11, TFAM, POLRMT, or TFB2M to allow for immunoprecipitation of the respective protein-DNA complexes. The antibody-bound complexes were captured using protein A/G-coated magnetic beads, and after thorough washing to remove non-specifically bound DNA, the eluted DNA was purified for subsequent analysis. Quantitative real-time PCR was performed using primers specifically targeting the mtDNA D-loop region (forward: CACCCCTCACCCACTAGGA TAC; reverse: TCCATGGGGACGAGAAGGGATT) to determine the enrichment of each protein's association with this regulatory region. The degree of enrichment was calculated as the percentage of immunoprecipitated DNA relative to the input DNA amount.

Statistical analysis

For statistical analysis, the study employed the Student's t-test to evaluate the experimental data. Statistically significant differences were denoted using the following conventions: ns (not significant) for $p \geq 0.05$, indicating a lack of statistically significant difference; * for $p < 0.05$, representing a statistically significant difference; ** for $p < 0.01$, denoting a highly significant difference; and *** for $p < 0.001$, representing an extremely significant difference. All data were reported as mean ± standard deviation (SD).

Results

Elevated expression of HOXC11 associates with poor prognosis in CRC patients

To investigate the potential role of HOXC11 in CRC, we initially examined its expression levels in CRC tissues. Firstly, we downloaded transcriptome sequencing data from The Cancer Genome Atlas (TCGA) database, including tumor tissues from CRC patients and normal colon tissues. Analysis of HOXC11 expression revealed a significant upregulation in CRC tissues compared to normal colon tissues (Fig. 1A). Subsequently, we validated

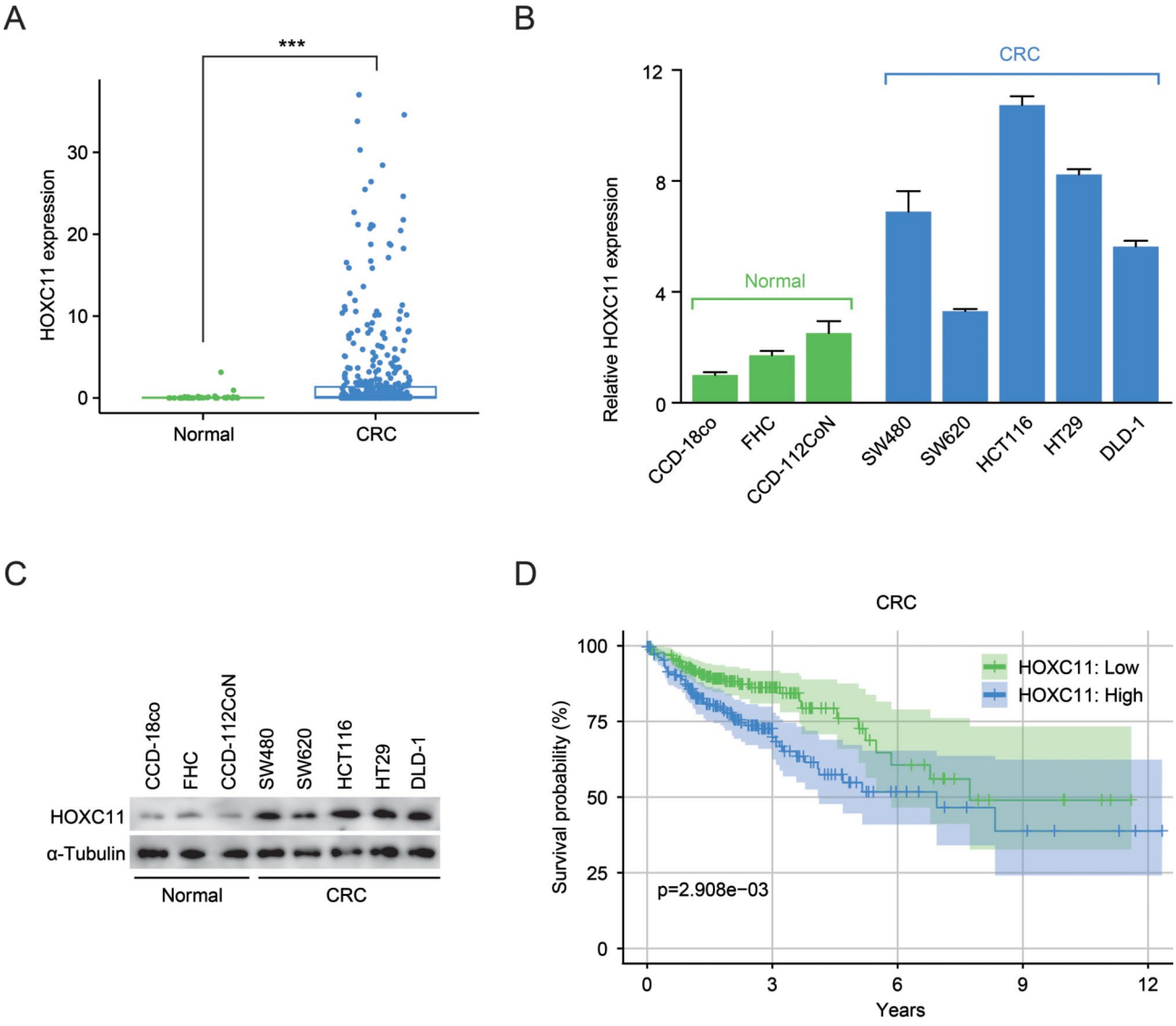

Fig. 1 Upregulation of HOXC11 expression in CRC and its association with reduced overall survival. (**A**) Boxplot showing the expression levels of HOXC11 in colorectal cancer samples and normal samples obtained from The Cancer Genome Atlas (TCGA) database. (**B-C**) Real-time PCR (**B**) and Western blot (**C**) analysis of HOXC11 expression in different normal colorectal epithelial cells and CRC cells. (**D**) Kaplan-Meier plot analysis of the relationship between HOXC11 expression levels and overall survival of CRC patients

this finding in human cell lines via assessing the mRNA and protein levels of HOXC11 in various normal colon epithelial cell lines and CRC cell lines using real-time PCR and western blotting analysis. The results showed a significant increase in HOXC11 mRNA (Fig. 1B) and protein levels (Fig. 1C) in CRC cell lines compared to normal colon epithelial cells.

Furthermore, to investigate whether high HOXC11 expression correlates with prognosis in CRC patients, we performed Kaplan-Meier survival analysis and observed a significant association between high HOXC11 expression and shorter overall survival in CRC patients (Fig. 1D), suggesting that elevated HOXC11 expression indicates poorer prognosis in CRC patients.

Reversal of acquired chemoresistance by HOXC11 knockout in CRC cells

Next, we aimed to investigate whether the loss of HOXC11 could reverse acquired chemoresistance in CRC cells. To this end, we established CRC cell lines resistant to 5FU, irinotecan, or oxaliplatin through long-term low-dose drug induction. By assessing the sensitivity of the drug-resistant cells and their parental counterparts to the three chemotherapy drugs, we observed a substantial decrease in sensitivity to chemotherapy drugs in the drug-resistant cell lines compared to their parental cells (Fig. 2A-F), indicating the successful establishment of drug-resistant cell lines. Next, we proceeded to knock out HOXC11 using CRISPR-Cas9 technology

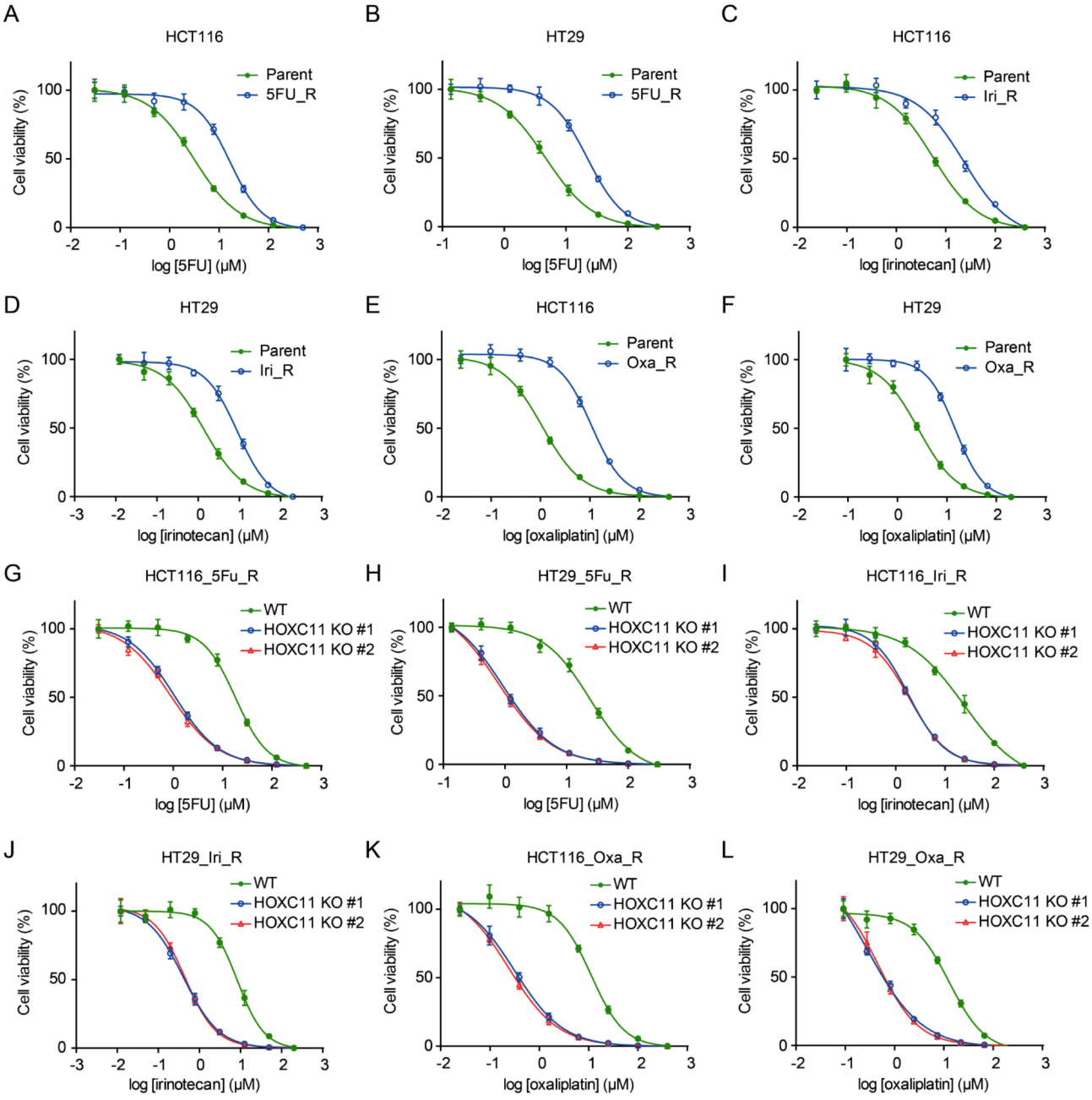

Fig. 2 Effective reversal of CRC cell chemoresistance by HOXC11 knockout. (**A-F**) Sensitivities of drug-resistant HCT116 and HT29 cell lines, along with their parental cell lines, to chemotherapyy drugs were evaluated using the sulforhodamine B (SRB) assay. (**G-L**) Cell viability of WT and HOXC11 knockout drug-resistant cells upon treatment with different concentrations of 5FU (**G** and **H**), irinotecan (**I** and **J**), or oxaliplatin (**K** and **L**) was determined using the SRB assay

in the drug-resistant cells and compared the sensitivity to chemotherapy drugs between cells with intact HOXC11 (WT) and those with knocked-out HOXC11. We observed a significant increase in sensitivity to chemotherapy drugs in the cells lacking HOXC11, indicating that the loss of HOXC11 can effectively reverse acquired chemoresistance in CRC cells (Fig. 2G-L). Together, these results demonstrate that knocking out HOXC11 effectively reverses acquired chemoresistance.

Extranuclear localization of HOXC11 drives chemoresistance in CRC cells

To investigate the molecular mechanisms underlying HOXC11-mediated acquired chemoresistance in CRC, we employed immunofluorescence staining to examine the subcellular localization and expression of HOXC11 in normal and chemoresistant CRC cells. Results revealed that while HOXC11 primarily localized to the nucleus in normal CRC, intriguingly, in chemoresistant CRC cells,

HOXC11 was not only present in the nucleus but also distributed outside the nucleus (Fig. 3A). Thus, we hypothesized that extranuclear HOXC11 might be the critical regulator mediating CRC chemoresistance. To validate this hypothesis, we ectopically introduced HOXC11 fused with N-terminal nuclear localization signal (NLS) or nuclear export signal (NES), along with C-terminal 6xHis tag (NLS-HOXC11-6xHis or NES-HOXC11-6xHis), into HOXC11 knockout chemoresistant cells using lentiviral-mediated transduction. Immunofluorescence analysis revealed that exogenous NLS-HOXC11-6xHis specifically localized to the nucleus (Figure S1), while NES-HOXC11-6xHis was exclusively present outside the nucleus (Figure S1). By employing this approach, we successfully generated CRC cell lines expressing HOXC11 exclusively in the nucleus or extranuclear compartments. Further assessment comparing HOXC11 knockout cells, cells expressing NLS-HOXC11-6xHis, and cells expressing NES-HOXC11-6xHis revealed that exogenous expression of NES-HOXC11-6xHis markedly reduced cell sensitivity to chemotherapy drugs (Fig. 3B-G), thereby reversing the chemosensitizing effect mediated by HOXC11 deficiency in resistant cells. In contrast, the expression of NLS-HOXC11-6xHis had no effect on chemosensitivity (Fig. 3B-G). These findings confirm that extranuclear localization of HOXC11 is crucial for regulating CRC cell chemoresistance.

Mitochondrial HOXC11 subpopulation regulates chemoresistance in CRC cells

Next, we explored the extranuclear localization of HOXC11 using immunofluorescence staining. Interestingly, we observed significant co-localization between HOXC11 and the mitochondria-specific dye MitoTracker (Fig. 4A and B), as well as the mitochondrial marker protein TFAM (Figure S2 A and B). These findings indicate that HOXC11 subpopulations outside the nucleus were present specifically within the mitochondria. Furthermore, we compared the mitochondrial respiratory function between normal and chemoresistant CRC cells and observed a significant increase in mitochondrial respiratory function in chemoresistant cells (Fig. 4B and C). Given the well-established role of mitochondrial respiratory function in mediating chemotherapy resistance [11–13], we hypothesized that the mitochondrial HOXC11 subpopulation may influence the chemoresistance of CRC cells by regulating mitochondrial respiratory function. To validate this hypothesis, we first compared the mitochondrial respiratory function between HOXC11 knockout chemoresistant cells and their counterparts with intact HOXC11 expression, revealing a significant reduction in mitochondrial respiratory function upon HOXC11 knockout (Fig. 4D-I). Furthermore, to further investigate the role of the mitochondrial HOXC11 subpopulation in mediating chemoresistance, we utilized lentiviral-mediated gene delivery to introduce HOXC11 fused with a N-terminal mitochondrial targeting sequence (MTS) and a C-terminal 6xHis tag (MTS-HOXC11-6xHis) into HOXC11 knockout chemoresistant cells. Immunofluorescence staining confirmed the specific localization of exogenously expressed MTS-HOXC11-6xHis within the mitochondria (Figure S3). Through comparative analysis of HOXC11 knockout cells and cells expressing MTS-HOXC11-6xHis, we observed a significant reduction in the chemosensitivity of HOXC11 knockout cells upon exogenous expression of MTS-HOXC11-6xHis (Fig. 5A-F), indicating that specific overexpression of the mitochondrial HOXC11 subpopulation effectively reversed the chemosensitization mediated by HOXC11 knockout. These findings collectively demonstrate that the mitochondrial HOXC11 subpopulation mediates chemoresistance in CRC cells by promoting mitochondrial respiratory function.

HOXC11 modulates mitochondrial respiratory function through transcriptional regulation of mtDNA

We further investigated the molecular mechanism by which HOXC11 regulates mitochondrial respiratory function in CRC cells. Given the well-known role of HOXC11 as a transcription factor in regulating gene expression [15, 16], we hypothesized that the mitochondrial subpopulation of HOXC11 may also participate in the transcriptional regulation of mitochondrial genes. To test this hypothesis, we assessed the expression levels of mitochondrial genes in chemoresistant cells with intact and knocked-out HOXC11. We found that the depletion of HOXC11 in chemoresistant CRC cells significantly decreased the overall expression levels of mitochondrial genes (Fig. 6A-C). Importantly, the knockout of HOXC11 did not affect the copy number of mtDNA (Fig. 6D), ruling out the possibility that HOXC11 influences the expression of mitochondrial genes by affecting mtDNA copy number. Furthermore, using mtDNA immunoprecipitation assay, we observed a high enrichment of HOXC11 at the D-loop regulatory region of mtDNA, which was markedly reduced upon HOXC11 knockout (Fig. 6E). Additionally, we found that the depletion of HOXC11 significantly decreased the enrichment of components of the mtDNA transcriptional regulatory complex, including TFAM (Fig. 6F), POLRMT (Fig. 6G), and TFB2M (Fig. 6H), at the D-loop region of mtDNA. These findings demonstrate that HOXC11 regulates mitochondrial respiratory function by participating in the transcriptional regulation of mtDNA.

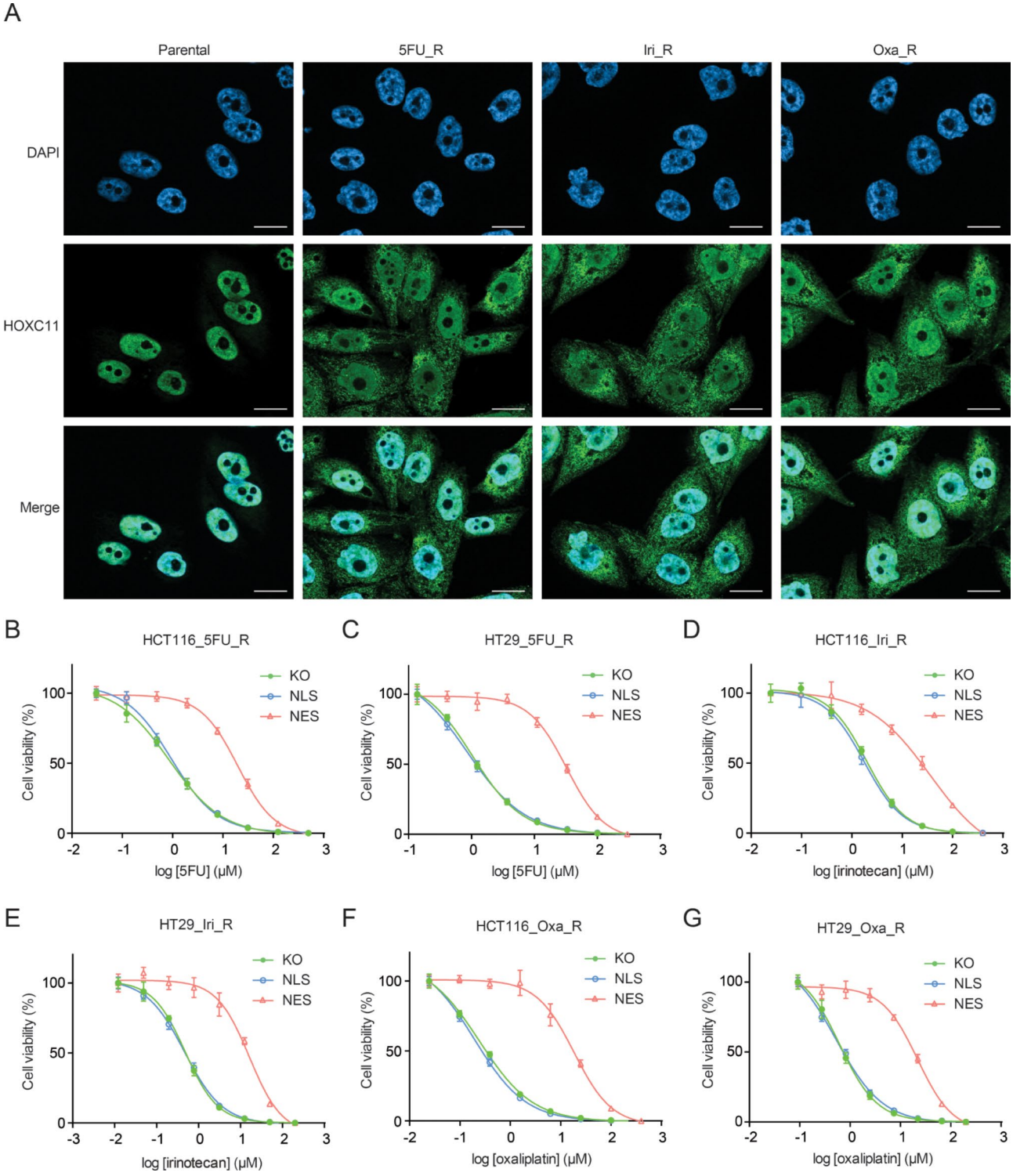

Fig. 3 Chemoresistance in CRC cells is regulated by HOXC11 residing outside the nucleus. (**A**) Immunofluorescence staining was employed to assess the subcellular localization and expression of HOXC11 in normal HCT116 cells and their drug-resistant counterparts. Scale bar represents 20 μm. (**B-G**) Lentiviral-mediated ectopic expression of HOXC11, fused with N-terminal nuclear localization signal (NLS) or nuclear export signal (NES) sequences and C-terminal 6xHis tag, in HOXC11-knockout and drug-resistant HCT116 and HT29 cells. Sensitivity of these cells to 5FU (**B** and **C**), irinotecan (**D** and **E**), and oxaliplatin (**F** and **G**) was evaluated using the SRB assay

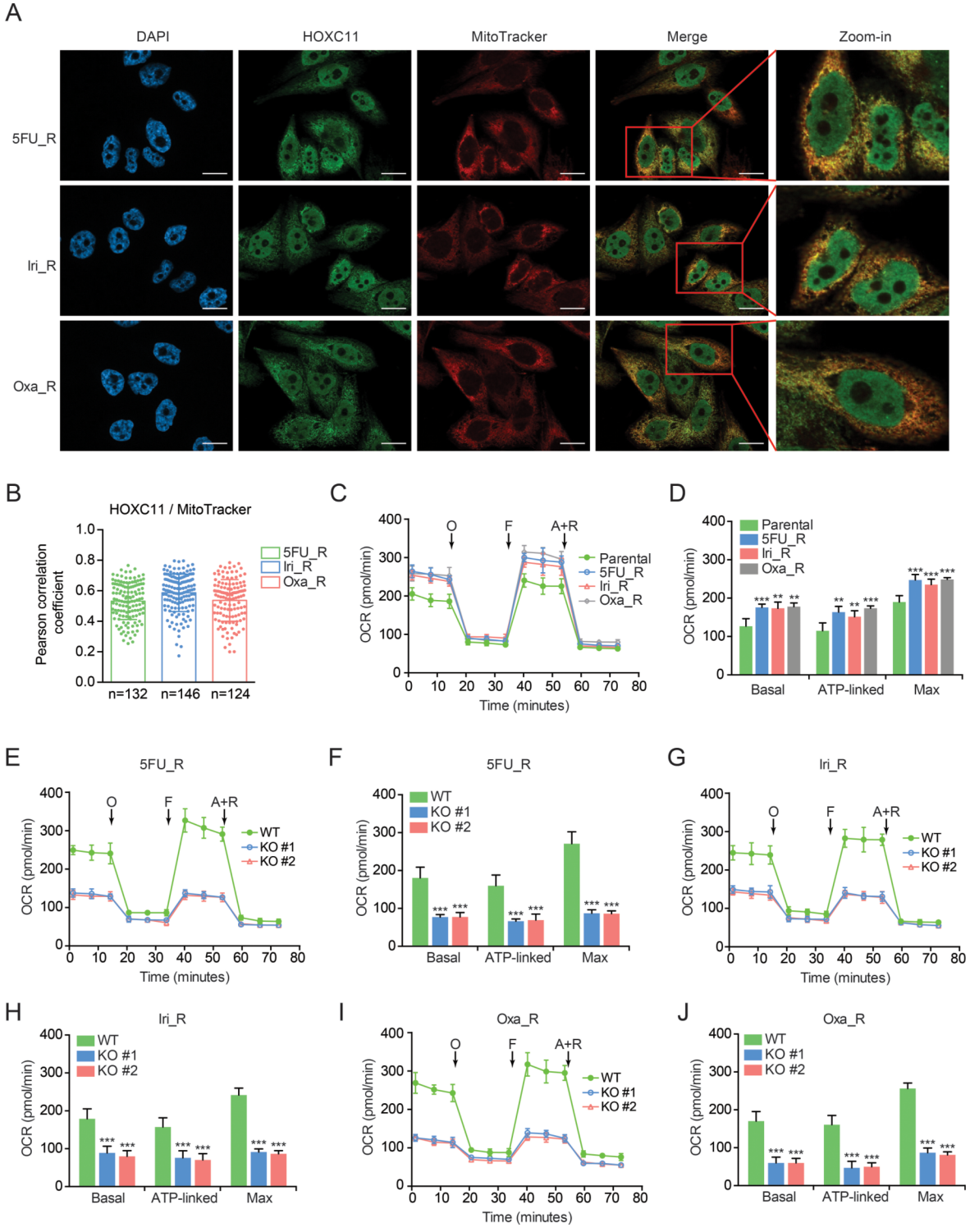

Fig. 4 HOXC11 localizes to mitochondria and regulates mitochondrial respiratory function in drug-resistant cells. (**A**) Immunofluorescence staining reveals the localization of HOXC11 within the mitochondria of chemoresistant HCT116 cells. Scale bar represents 20 μm. (**B**) Pearson correlation analysis was conducted to determine the co-localization of HOXC11 and mitochondria (stained by MitoTracker) in the indicated cells, as previously reported [35, 36]. Each dot represents an individual cell, with the sample size (n) indicated in the figure. (**C** and **D**) Mito stress test was employed to determine the mitochondrial respiratory function levels in both normal and chemoresistant HCT116 cells. (**E-J**) Seahorse mito stress test was utilized to analyse the mitochondrial respiratory function levels of chemoresistant HCT116 cells with or without HOXC11 knockout. (**C-J**) Data are presented as mean + SD. Statistical analysis was performed using t-test, comparing with parental (**C** and **D**) or WT (**E-J**) cells. Significance levels are denoted as ** for $p < 0.01$ and *** for $p < 0.001$

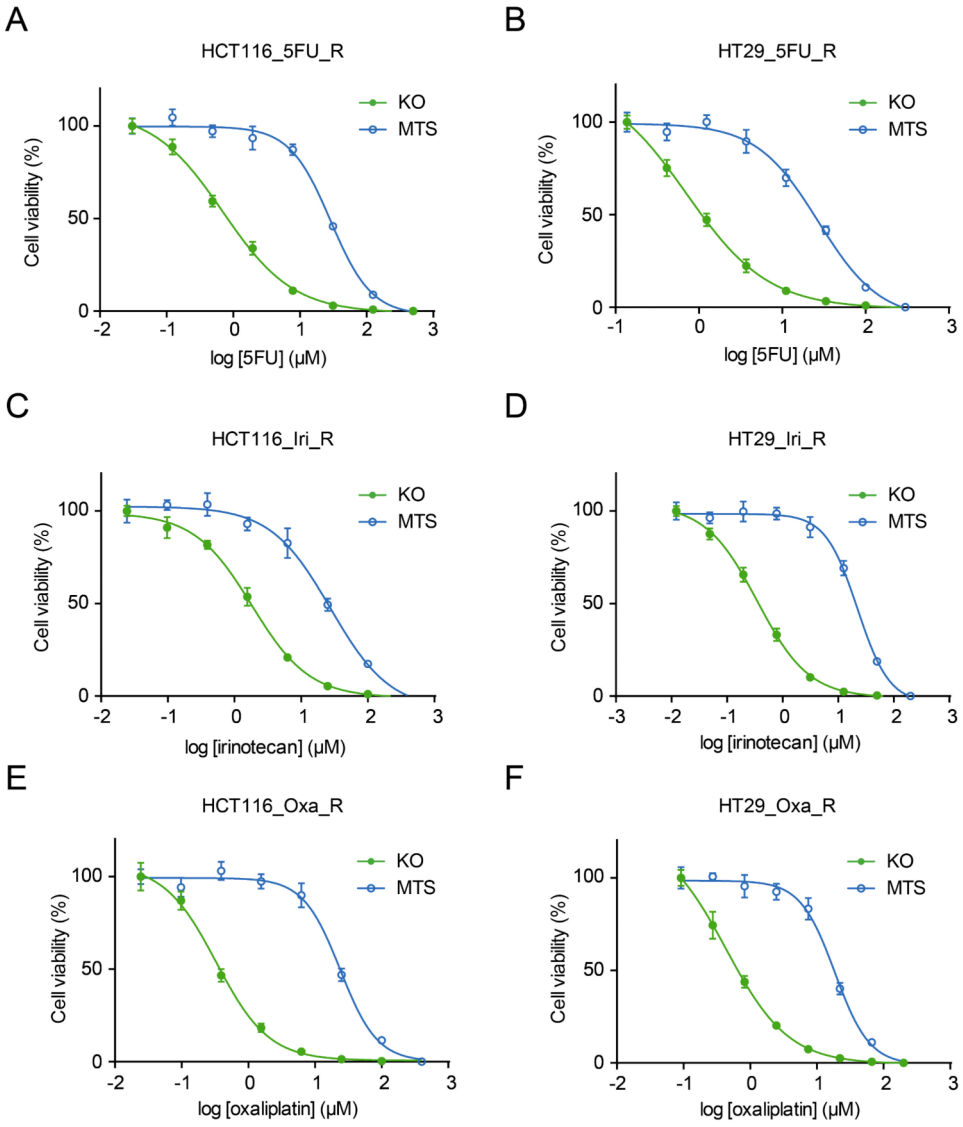

Fig. 5 Exogenous expression of mitochondrial HOXC11 reverses chemotherapy sensitization caused by HOXC11 deletion. (**A-F**) Lentiviral-mediated ectopic expression of HOXC11, fused with a mitochondrial targeting sequence (MTS) at the N-terminus and a 6xHis tag at the C-terminus, was performed in HOXC11 knockout drug-resistant HCT116 or HT29 cells. Subsequently, the sensitivity to 5FU (**A** and **B**), irinotecan (**C** and **D**), and oxaliplatin (**E** and **F**) was assessed in HOXC11 knockout cells and those expressing only MTS-HOXC11 using the SRB assay

Inhibition of mtDNA transcription reverses chemoresistance in CRC cells independent of HOXC11 expression

To further explore the impact of HOXC11-mediated mtDNA transcriptional regulation on chemoresistance in CRC cells, we investigated whether direct inhibition of mtDNA transcription could effectively reverse chemoresistance. To achieve this, we employed a widely used mtDNA transcription inhibitor, IMT1B [32, 33], to directly suppress mtDNA transcription in chemoresistant CRC cells, and subsequently evaluated the effect of IMT1B on the chemosensitivity. The results revealed that IMT1B significantly increased the sensitivity of chemoresistant CRC cells to chemotherapy agents (Fig. 7A-F), effectively reversing the acquired chemoresistance. Importantly, the chemosensitizing effect mediated by IMT1B could not be reversed by ectopic overexpression of NES-HOXC11 or MTS-HOXC11 (Fig. 7A-F). In fact, treatment with IMT1B led to a significant decrease in the expression levels of mtDNA genes in chemotherapy-resistant CRC cells, without affecting the expression level of HOXC11 (Fig. 7G). This observation further suggests that IMT1B does not exert its effect by modulating HOXC11 expression, but rather, consistent with previous reports [32, 33], by directly targeting and inhibiting mitochondrial gene transcription. Taken together, these findings demonstrate that the transcriptional regulation

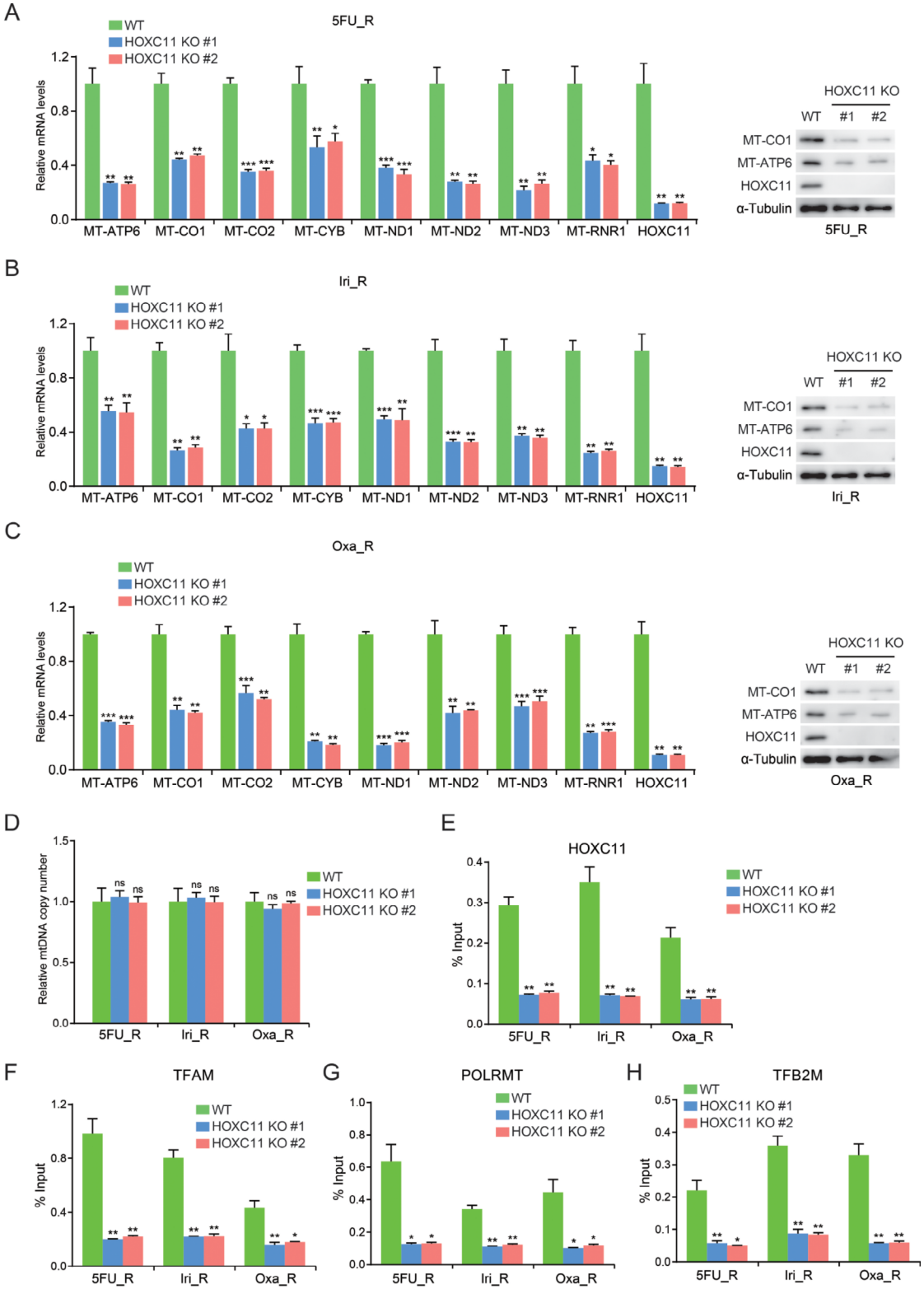

Fig. 6 Impact of HOXC11 knockout on mitochondrial gene expression and transcription regulation. (**A-C**) Real-time PCR (left panel) and western blot (right panel) analyses of mitochondrial gene expression levels in drug-resistant HCT116 cells with or without HOXC11 knockout. (**D**) Real-time PCR analysis of mtDNA copy number in drug-resistant HCT116 cells with or without HOXC11 knockout. (**E-H**) Enrichments of HOXC11 (**E**), TFAM (**F**), POLRMT (**G**), and TFB2M (**H**) in the transcription regulatory region of mtDNA (D-loop) were determined using mtDNA immunoprecipitation assay in WT and HOXC11-knockout drug-resistant cells. (A-H) Data are presented as mean + SD, and statistical significance was determined using t-test comparing with the WT group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

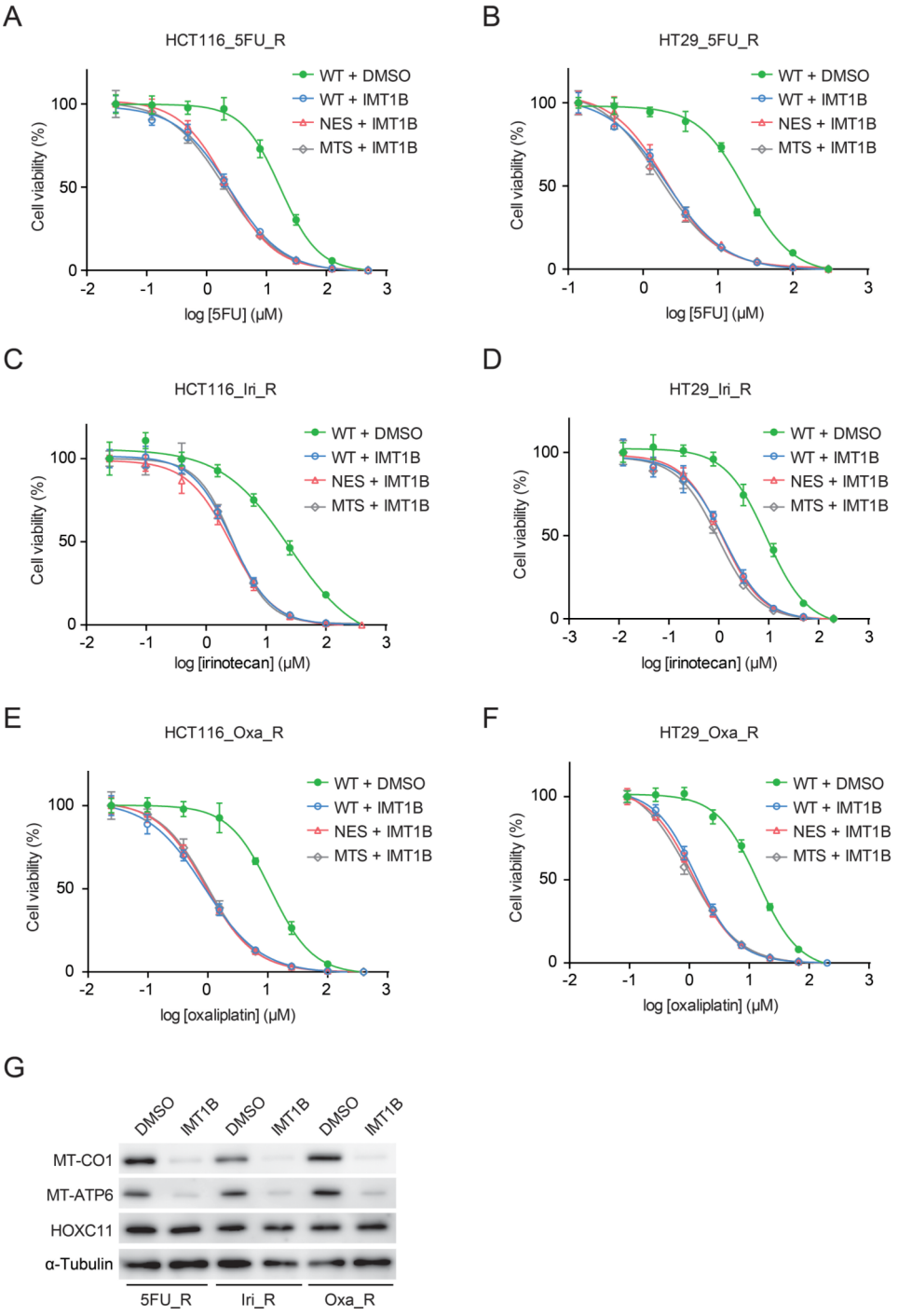

Fig. 7 MtDNA transcription inhibitor IMT1B reverses chemotherapy resistance in CRC cells. (**A-F**) Drug-resistant HCT116 and HT29 cells were transduced with lentiviral vectors to ectopicalyly express NES-HOXC11 and MTS-HOXC11. Subsequently, the sensitivity of these cells to 5FU (**A** and **B**), irinotecan (**C** and **D**), and oxaliplatin (**E** and **F**) was assessed using the SRB assay upon treatment with IMT1B. HCT116 and HT29 cells were treated with 2.5 μM and 1 μM IMT1B, respectively. (**G**) Western blot analysis of mitochondrial genes MT-CO1, MT-ATP6, and HOXC11 expression levels in chemotherapy-resistant HCT116 cells treated with or without 2.5 μM IMT1B

of mtDNA serves as a downstream effector of HOXC11 to mediate the chemoresistance of CRC cells.

HOXC11 expression correlates with poor prognosis in chemotherapy-treated CRC patients

To further explore whether the chemoresistance mediated by HOXC11 could be observed in CRC patient data, we conducted an analysis of TCGA CRC transcriptome data and corresponding clinical information. Patients were stratified based on their chemotherapy treatment status into four groups: 5FU treatment group, irinotecan treatment group, oxaliplatin treatment group, and untreated group. Kaplan-Meier survival analysis was then performed to assess the impact of HOXC11 expression levels on the overall survival duration of patients in each group. The results showed that high expression of HOXC11 was significantly associated with shorter survival in CRC patients who received chemotherapy treatment, including those in the 5FU, irinotecan, and oxaliplatin treatment groups (Fig. 8A-C). However, in CRC patients who did not receive any medication, the expression level of HOXC11 did not significantly affect the overall survival period (Fig. 8D). These findings indicate that high expression of HOXC11 may serve as a potential poor prognostic indicator specifically in CRC patients undergoing chemotherapy treatment.

Discussion

HOXC11 is a member of the homeobox gene family, which encodes transcription factors that play pivotal roles in various malignancies [14–18]. In breast cancer, HOXC11 overexpression, which correlates with poor prognosis, contributes to cancer progression and endocrine therapy resistance through its interaction with the steroid receptor coactivator SRC-1, leading to the repression of differentiation genes and apoptosis regulators [15, 34]. HOXC11 overexpression in lung adenocarcinoma promotes tumor progression by transcriptionally

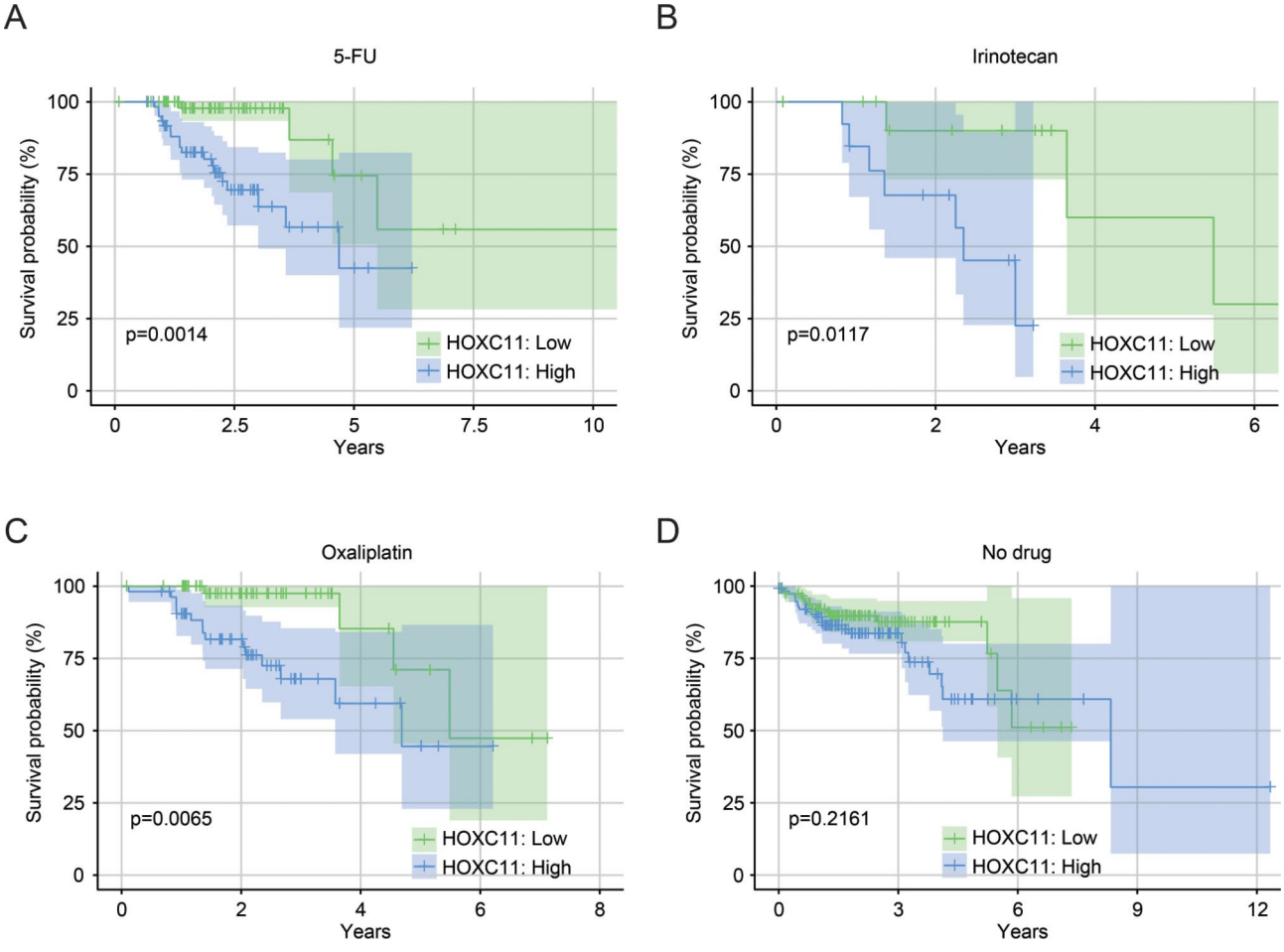

Fig. 8 High HOXC11 expression correlates with shorter overall survival specifically in chemotherapy-treated CRC patients. (**A-D**) CRC patients were categorized based on whether they received chemotherapy treatment: 5FU-treated group (**A**), irinotecan-treated group (**B**), oxaliplatin-treated group (**C**), and control group treated with no drug (**D**). Kaplan-Meier survival analysis was performed to assess the impact of HOXC11 expression levels on the overall survival of CRC patients across various treatment cohorts. Patients were stratified into high and low expression groups based on their HOXC11 expression levels. Statistical significance was determined using the log-rank test, with *p*-values less than 0.05 indicating significant differences

upregulating SPHK1, thereby enhancing proliferation and facilitating cancer progression [16]. In clear cell renal cell carcinoma and gastric cancer, HOXC11 overexpression induces cellular proliferation and is associated with poor prognosis, highlighting its oncogenic role and potential as a prognostic biomarker in these malignancies [14, 17]. Our study extends the current understanding of HOXC11's role in cancer by demonstrating its functional significance in mediating CRC chemoresistance.

Intriguingly, our mechanistic investigations revealed a novel extranuclear role for HOXC11 in regulating chemoresistance. We observed that a functional subset of HOXC11 localizes to the mitochondria in chemoresistant CRC cells. This finding is particularly significant, as mitochondria have emerged as key players in cancer chemoresistance [11–13]. By modulating mitochondrial respiratory function, cancer cells can enhance their survival and evade chemotherapy-induced cell death [11–13]. Our study demonstrates that the mitochondrial subpopulation of HOXC11 directly regulates mitochondrial respiratory function by participating in the transcriptional regulation of mtDNA. This novel mechanism expands our understanding of how HOXC11 contributes to chemoresistance in CRC.

Furthermore, we showed that direct inhibition of mtDNA transcription using a specific inhibitor, IMT1B, effectively reverses chemoresistance in CRC cells, independent of HOXC11 expression. This finding highlights the potential therapeutic implications of targeting mitochondrial function in CRC treatment. By suppressing mtDNA transcription, we can potentially sensitize chemoresistant CRC cells to chemotherapy, thereby improving patient outcomes.

However, despite the novel insights provided by this study, several limitations should be acknowledged. One of the limitations of our study is its reliance primarily on cell-based experiments, which lack in vivo validation through animal models. Future studies incorporating animal models will be essential to validate our findings and assess the translational relevance of targeting HOXC11 as a therapeutic strategy for overcoming chemoresistance in CRC. Additionally, such experiments could provide valuable insights into the systemic effects of HOXC11 modulation and its impact on tumor progression and treatment outcomes in a more physiologically relevant context.

Another limitation of the study is the lack of clarity regarding the specific mechanism by which HOXC11 localizes to mitochondria in chemoresistant cells. Future investigations aimed at unraveling the precise mechanisms responsible for targeting HOXC11 to mitochondria in chemoresistant cells will be essential for a comprehensive understanding of HOXC11-mediated chemoresistance in CRC. Addressing this gap in knowledge will not only enhance our understanding of the role of HOXC11 in CRC but also provide valuable insights into potential therapeutic strategies targeting mitochondrial dynamics to overcome chemoresistance.

In conclusion, our study identifies HOXC11 as a novel regulator of chemoresistance in CRC cells through the modulation of mitochondrial respiratory function. We demonstrate that the mitochondrial subpopulation of HOXC11 directly participates in the transcriptional regulation of mtDNA, thereby influencing chemoresistance. These findings provide new insights into the molecular mechanisms underlying CRC chemoresistance and highlight the potential therapeutic implications of targeting mitochondrial function in CRC treatment. Further investigations into the HOXC11-mitochondrial axis may lead to the development of novel strategies to overcome chemoresistance and improve patient outcomes in CRC.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Author contributions

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Data availability

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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