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FULL LENGTH ARTICLE

Ubiquitin ligase DTX3 empowers mutant p53 to promote ovarian cancer development

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Abstract The deltex family protein DTX3 is believed to possess E3 ubiquitin ligase activity, as it contains a classic RING finger domain. However, its biological role and the underlying mechanism in cancer remain largely elusive. Here, we identified DTX3 as a novel mutant p53-interacting protein in ovarian carcinoma. Mechanistically, DTX3 mediated mutant p53 ubiquitination and stabilization by perturbing the MDM2-mutant p53 interaction, consequently leading to activation of diverse mutant p53 target genes. Importantly, a positive correlation between the expression of DTX3 and mutant p53 target genes was further validated in ovarian carcinomas. Ectopic DTX3 promoted, while depletion of DTX3 suppressed, ovarian cancer cell proliferation and invasion. Remarkably, the pro-tumorigenic effect of DTX3 is dependent on mutant p53, because ablation of mutant p53 significantly impaired DTX3-induced gene expression and ovarian cancer cell growth and propagation. Furthermore, DTX3 elevated the

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expression of mutant p53 target genes and boosted ovarian tumor growth *in vivo*. Finally, DTX3 was amplified and overexpressed in ovarian carcinomas, which is significantly associated with unfavorable prognosis. Altogether, our findings unveil the oncogenic role of DTX3 in ovarian cancer development by bolstering mutant p53 activity.

Introduction

Epithelial ovarian cancer (EOC) is one of the leading causes of cancer-related death in women worldwide. The frontline treatment includes primary debulking surgery followed by chemotherapy, whereas most patients die from the relapsed disease that eventually progresses to a more aggressive stage.[1] Although our understanding of the etiology and pathology of EOC has been advanced, the overall survival of the disease has not been significantly improved for decades.[2] Thus, systematic dissection of the mechanisms behind ovarian carcinogenesis, progression, and refractoriness remains an active area of investigation.

Mutation of the tumor suppressor gene *TP53* is both an early event and invariable characteristic of high-grade serous ovarian carcinoma (HGSOC) that is the most common and aggressive histotype of EOC.[3–5] The *TP53*-encoded protein p53 is regarded as the "guardian of the genome" to prevent cells from malignant transformation[6,7] by transcriptionally modulating the expression of a cluster of genes, such as *CDKN1A*, *BTG2*, and *GADD45A*, involved in cell cycle arrest and DNA repair.[8] Moreover, p53 also eliminates cancer cells by driving irreversible cellular outcomes, including cell senescence, apoptosis, and ferroptosis.[9,10] Mutation of *TP53*, which is associated with cancer initiation and development, not only abrogates or counteracts the tumor suppressive activity of wild-type p53 (wtp53), but also endows mutant p53 (mtp53) with oncogenic function, namely "gain-of-function" (GOF), resulting in enhanced cancer cell survival, proliferation, and metastasis.[11,12] Various working models have been proposed accounting for mtp53's GOF. As a vast majority of missense mutations occur in the DNA-binding domain (DBD),[13] mtp53 proteins are usually deprived of the ability to associate with the canonical p53-responsive DNA elements. Nevertheless, mtp53 can indirectly bind to different DNA elements and regulate gene transcription via interaction with other transcription factors.[14] For instance, CDK4/Cyclin D mediates phosphorylation of mtp53-R249S, a liver cancer-derived hotspot p53 mutant, which translocates to the nucleus and promotes rDNA transcription by interacting with c-MYC.[15,16] In the rare case, mtp53-R273P was found to associate with the specifically structured DNA, such as matrix attachment regions (MARs), to induce gene transcription.[17] In addition, some p53 mutants, such as R248W and R273H, were shown to execute GOF by binding to proteins unrelated to transcription and modulating their functions.[18] In ovarian cancer, mtp53 was considered a driver for cancer onset and progression,[5,19] as well as a biomarker for targeted therapy.[20–22] Recently, we also revealed that mtp53-S241F and R273H endorse ovarian cancer development by transcriptionally activating a myriad of genes associated with cell survival, proliferation, metastasis, and metabolism.[23] Because of the complex interplays between mtp53 and its partnerships, additional efforts are needed to delineate the mtp53 network in ovarian cancer, which would provide useful information for future development of therapeutic approaches.

In our attempt to explore mtp53-interacting proteins in primary ovarian cancer tissues as previously described,[23] the deltex E3 ubiquitin ligase 3 (DTX3), also known as RNF154, was revealed as an additional mtp53-binding partner that is further detailed below. *Deltex* (*dx*) was initially identified as a positive regulator of Notch function in *Drosophila*.[24,25] Mammalian homologues of *Deltex*, including DTX1 to 4 and a distantly related protein DTX3L, compose the DTX protein family that shares a highly conserved C-terminal region harboring a classic RING finger domain.[26] DTX1, DTX2, and DTX4 were shown to interact with the intracellular ankyrin repeats of NOTCH via the N-terminal basic domains,[26,27] leading to deregulation of the Notch signaling in embryogenesis, lymphocyte development, and several types of cancer.[28–32] Nevertheless, the understudied member DTX3 was believed unable to bind to NOTCH, owing to lack of the N-terminal module critical for the interaction between DTX proteins and NOTCH receptors.[26,27,32] An integrated genomic screening showed that DTX3 is amplified in the luminal-subtype of breast cancer, which is associated with highly proliferative luminal tumors and poor prognosis.[33] However, the biological function and molecular basis of DTX3 during cancer development remain largely elusive.

In this study, we identified DTX3 as a mtp53-interacting protein through a co-immunoprecipitation (IP) assay coupled with mass spectrometry (MS) analysis as described.[23] This interaction was further validated in cancer cells. DTX3 mediated mtp53 poly-ubiquitination and protein stability, leading to induction of diverse mtp53 target genes that are required for cancer cell proliferation and metastasis. Consistently, we showed that ectopic expression of DTX3 prompts, whereas ablation of DTX3 inhibits, ovarian cancer cell growth and invasion in a mtp53-dependent fashion. Finally, DTX3 endorsed ovarian tumor growth *in vivo* and was associated with unfavorable prognosis of ovarian cancer. Hence, this study unveils DTX3 as an oncoprotein that can promote mtp53 activity in ovarian cancer.

Materials and methods

Cell culture and transient transfection

Human cancer cell lines ES-2, OVCA420, SKOV3, HCT116$^{p53^{-/-}}$were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. All cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Cells were seeded on plates at appropriate density 12—24 h before transfection, and then transfected with plasmids or siRNAs using Hieff Trans™ liposomal transfection reagent following the manufacturer's protocol (Yeasen, Shanghai, China). Cells were harvested or collected at 36—48 h post-transfection for future experiments. Proteasome inhibitor MG132 (Sigma—Aldrich St. Louis, MO, USA) treatment was conducted 4—6 h before cell harvest.

Plasmids and antibodies

The Flag-tagged DTX3-expressing plasmid was purchased from Vigene Biosciences, Inc. (Shandong, China). The Myc-tagged DTX3 plasmid was generated by inserting the full-length cDNA amplified by PCR into the pcDNA3.1/Myc-His vector, using the following primers, 5′-GGAATTCATGTCGTTCGTCCTGTCC-3′ and 5′-CGGGATCCGTCATCTGTGATACCCTTCGC-3'. The plasmids encoding non-tagged mtp53 (R175H, S241F, R248W, and R273H), His-Ub and HA-MDM2 were described previously.[23] The anti-Flag (Catalogue No. F1804, Sigma—Aldrich), anti-DTX3 (Catalogue No. ab197360, Abcam, Cambridge, MA, USA), anti-p53 (DO-1, Catalogue No. sc-126, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p53 (Catalogue No. ab179477, Abcam) anti-GAPDH (Catalogue No. 60004-1-Ig, Proteintech, Hubei, China), anti β-actin (Catalog No. 60008-1-Ig, Proteintech), anti-Vinculin (Catalogue No. 66305-1-Ig, Proteintech), and the secondary antibodies for rabbit (Catalogue No. ARG65351, Arigo) and mouse (Catalogue No. ARG65350, Arigo), and the light chain-specific secondary mouse antibody (Catalogue No. 115-035-174, Jackson) were commercially purchased.

Reverse transcription and quantitative RT-PCR analysis

Total RNAs were isolated using the RNAiso Plus reagent (Takara, Japan) following the manufacturer's protocol. 0.5—1 μg total RNAs were used as templates for reverse transcription using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). Quantitative RT-PCR (RT-qPCR) was conducted using TB Green™ Premix according to the manufacturer's protocol (Takara, Japan). The primers for DTX3, PXN, IGFBP3, BUB1, MAP2K3, MYC, MMP13, MMP3, NCAPH, ITGA6, RANGAP1, BCL2L1, CCNA2, NFKB2, ARHGDIA, CENPA, CXCL1, ABCB1, CDC25C, MCM6, KIF20A, CDK1, DEPDC1, MAD1L1, and GAPDH cDNA detection were as follows: DTX3, 5′-TCGTTCGTCCTGTCCAGAATG-3′ and 5′-AAGTCTCGCCATCTATGAGGAT-3'; PXN, 5′-GGCTCTCCGTGCTCCCGAGTG-3′ and 5′-GCAGCAGGCGGTCGAGTT-3'; IGFBP3, 5′-AAAAAGCAGTGTCGCCCTTCC-3′ and 5′-TCCACATTAACCTTGCGGCAG-3'; BUB1, 5′-ATTCAAGCCACAGAGTGGAGCAG-3′ and 5′-AGAACTTGTGTTGGCAACCTTATGTG-3'; MAP2K3, 5′-CTGCGGTTCCCTTACGAGT-3′ and 5′-GCAATGTCCGTCTTCTTGGT-3'; MYC, 5′-GGAGATCCGGAGCGAATAG-3′ and 5′-CCTTGCTCGGGTGTTGTAAGT-3'; MMP13,5′-GAATTAAGGAGCATGGCGACT-3′ and 5′-CTAAGGAGTGGCCGAACT-3'; MMP3, 5′-ATCCTACTGTTGCTGTGCGTG-3′ and 5′-ACTTCTGCATTTCTCGGATTT-3'; NCAPH, 5′-AAACACGCAGATTACGGAACA-3′ and 5′-GTTGGTTGGTTCGGTGTCTTT-3'; ITGA6, 5′-GTCCAGAGCCAAGGTCCAG-3′ and 5′-CTCAATCGCCCATCACAAAA-3'; RANGAP1, 5′-GCTCCAAGGGTGCAGTTG-3′ and 5′-GCAGCATCCCTCTTGATTTC-3'; BCL2L1, 5′-GACTGAATCGGAGATGGAGACC-3′ and 5′-GCAGTTCAAACTCGTCGCCT-3'; CCNA2, 5′-AGCAGCCTGCAAACTGCAAAGTTG-3′ and 5′TGGTGGGTTGAGGAGAGAAACAC-3'; NFKB2, 5′-GGGGCATCAAACCTGAAGATTTCT-3′ and 5′-TCCGGAACACAATGGCATACTGT-3'; ARHGDIA, 5′-AGCCTGCGAAAGTACAAGGA-3′ and 5′-GGTCAGGCCAGTCACCAC-3'; CENPA, 5′-CTTCCTCCCATCAACACAGTCG-3′ and 5′-TGCTTCTGCTGCCTCTTGTAGG-3'; CXCL1, 5′-AGGGAATTCACCCCAAGAAC-3′ and 5′-ACTATGGGGGATGCAGGATT-3'; ABCB1, 5′-GGGAGCTTAACACCCGACTTA-3′ and 5′-GCCAAAATCACAAGGGTTAGCTT-3'; CDC25C, 5′-GTATCTGGGAGGACACATCCAGGG-3′ and 5′-CAAGTTGGTAGCCTGTTGGTTTG-3'; MCM6, 5′-GAGGAACTGATTCGTCCTGAGA-3′ and 5′-CAAGGCCCGACACAGGTAAG-3'; KIF20A, 5′-TCCTCAAGGAGTCACTGACAAG-3′ and 5′-GATGGGCCACTGACTGTTGT-3'; CDK1, 5′-CCTTGCCAGAGCTTTTGGAATACC-3′ and 5′-GACATGGGATGCTAGGCTTCCTGG-3'; DEPDC1, 5′-TGGGTATTATCTGCCATGAAGTGCCT-3′ and 5′-AGGTTGCAGCAAGCCCAAAATGT-3'; MAD1L1, 5′-TGGACTGGATATTTCTACCTCGG-3′ and 5′-CCTCACGCTCGTAGTTCCTG-3'; GAPDH, 5′-GGAGCGAGATCCCTCCAAAAT-3′ and 5′-GGCTGTTGTCATACTTCTCATGG-3'.

Immunoblotting

Cells were harvested and lysed in lysis buffer consisting of 50 mM Tris/HCl (pH7.5), 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and the protease inhibitor cocktail (Yeasen), and then incubated on ice for 45—60 min. Equal amounts of clear cell lysates (20—80 μg) were used for immunoblotting (IB) analysis as described previously.[34]

Immunoprecipitation

Immunoprecipitation (IP) was conducted using α-Flag or α-DO-1 antibodies as indicated in the figure legends. Briefly, ~500—1000 μg of proteins were incubated with the indicated antibody at 4 °C for 4 h or overnight. Protein A/G beads (Santa Cruz Biotechnology) were then added to the mixture that was further incubated at 4 °C for additional 1—2 h. Beads were washed at least three times with lysis buffer. Bound proteins and 10% inputs were analyzed by IB with antibodies as indicated in the figure legends.

In vivo ubiquitination assay

HCT116$^{p53^{-/-}}$ cells were transfected with combinations of plasmids encoding mtp53-R273H, His-Ub or Flag-DTX3 as indicated in the figure legends. MG132 was added to

cultures 4–6 h before harvest. At 48 h after transfection, cells were harvested and split into two aliquots, one for IB and the other for the ubiquitination assay. Briefly, cell pellets were lysed in buffer I (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0), 10 mM Tris–HCl (pH 8.0), 10 mM β-mercaptoethanol, 5 mM Imidazole) and incubated with Ni-NTA beads (Takara, Japan) that capture His-tagged proteins/complex at room temperature for 4–6 h. Beads were washed twice with buffer I, and twice with buffer II (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 6.3), 10 mM Tris–HCl (pH 6.3), 10 mM β-mercaptoethanol). The captured proteins were eluted and analyzed by IB with the indicated antibodies.

RNA interference

The siRNA against DTX3 were synthesized and purified by GenePharma (Shanghai, China). The siRNA sequences used were as follows: siDTX3-1, 5′-GGAACGACATCCACCACAAGA-3′, si-DTX3-2, 5′-GGCGGATGCTGGTCTCTAAGG-3′, and sip 53, 5′-GUAAUCUACUGGGACGGAA-3'. The siRNAs of 50–100 nM were transfected into cells using Hieff Trans™ liposomal transfection reagent following the manufacturer's protocol (Yeasen). Cells were harvested 48–72 h after transfection for IB or RT-qPCR.

Generation of stable cell lines

The lentivirus-based pLenti-EF1a-EGFP-Puro-CMV plasmids encoding DTX3 and mtp53-R273H were purchased from Vigene Biosciences (Shandong, China) and OBio Technology (Shanghai, China), respectively. The recombinant viruses were packaged as described[35] and infected cells with appropriate amounts for overnight. The stable cells were selected with 1 μg/ml puromycin.

Cell viability assay

To assess the long-term cell survival, the Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) was used according to the manufacturer's instructions. Cell suspensions were seeded at 3000 cells per well in 96-well culture plates at 12 h post-transfection. CCK-8 at a final concentration of 10% was added to the cultures for 2–4 h, and the absorbance of the samples was measured at 450 nm using a Microplate Reader every 24 h.

Transwell invasion assay

The invasion assay was performed using Transwell chamber inserts in a 24-well plate as described.[23] Briefly, 5×10^4 cells suspended in 100 μL of serum-free medium were added to the upper chamber. The lower chambers were filled with the normal culture medium. The cells were cultured for 24–36 h at 37 °C. Afterward, the cells on the upper surface were scraped and washed away, and the cells on the lower surface were fixed with methanol and stained with 0.1% crystal violet. The number of invaded cells was counted in at least five randomly selected fields under an optical microscope by image J software.

Flow cytometry analysis

The PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) was used for apoptosis analysis according to the manufacturer's instruction. Briefly, cells were washed twice with cold PBS, resuspended in binding buffer, and stained with PE Annexin V and 7-AAD for 15 min at RT. The cells were then analyzed by a FC500 MPL flow cytometer (Beckham coulter, Indianapolis, IN, USA).

Mouse xenograft experiment

Female BALB/c nude mice of five weeks were purchased from the Department of Laboratory Animal Science in Shanghai Medical College of Fudan University. To evaluate the effect of DTX3 overexpression on tumor growth *in vivo*, mice were subcutaneously inoculated with 5×10^5 ES-2 cells stably expressing pLent-vector or pLent-DTX3. Tumor growth was monitored every other day with electronic digital calipers in two dimensions. Tumor volume was calculated according to the formula: volume $=$ length $\times$ width^2 $\times$ 0.5. Mice were sacrificed by euthanasia and tumors were then harvested, weighed, and subjected to RT-qPCR analysis. The animal protocols were in compliance with ethical regulations and approved by the Animal Welfare Committee of Shanghai Medical College at Fudan University.

Database of cancer patients

The mRNA expression correlations between DTX3 and mtp53 target genes were evaluated by the online tool GEPIA (http://gepia.cancer-pku.cn) which includes data from TCGA and GTEx database using Pearson correlation coefficient. The Oncomine database (https://www.oncomine.org) was used to analyze the DTX3 gene copy number and mRNA level in cancerous versus normal tissues. Cancer patient survival was analyzed by the Kaplan–Meier method using the KM plotter database (kmplot.com/analysis/).

Statistics

All *in vitro* experiments were performed in biological triplicate. The Student's *t*-test or one way analysis of variance (ANOVA) was performed to evaluate the differences between two groups or more than two groups. The Cox univariate proportional hazards regression models was used to determine the independent clinical factors based on the investigated variables. Pearson's correlation was performed to analyze the correlation of the gene expression profiling. Asterisks represent statistical significance in the following way: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All the data are presented as mean ± SD.

Results

Identification of DTX3 as a mutant p53-interacting protein

To dissect the mechanism of mtp53 regulation, we conducted a proteomic approach to screen mtp53-binding proteins in ovarian cancer tissues,[23] resulting in the identification of the E3 ubiquitin ligase DTX3 as a new binding partner of mtp53. To validate this result, we performed a set of co-IP-IB assays to determine if DTX3 binds to mtp53 in cancer cells. As shown in Figure 1A—D, the exogenous mtp53 proteins, such as mtp53-R175H, S241F, R248W, and R273H, were co-immunoprecipitated with Flag-tagged DTX3 by the anti-Flag antibody. The results were then confirmed by the reciprocal co-IP analysis, as Flag-DTX3 could be co-immunoprecipitated with diverse p53 mutants, including mtp53-R175H, S241F, R248W, and R273H, using the anti-p53 antibody (Fig. 1E—H). Moreover, we verified the endogenous interaction of DTX3 and mtp53, as endogenous DTX3 could be co-immunoprecipitated with mtp53-S241F and R273H in ES-2 and OVCA420 cells, respectively (Fig. 1I, J). An additional ovarian cancer cell line SKOV3 stably expressing mtp53-R273H was also employed to confirm the DTX3-mtp53 interaction (Fig. 1K). Since many E3 ubiquitin ligases can target both mtp53 and wtp53[36], we tested if DTX3 binds to wtp53 as well. Indeed, both exogenous and endogenous interactions of the two proteins were detected by co-IP-IB analysis (Fig. S1). This observation suggested that DTX3 might also regulate wtp53 activity in other types of cancer with rare frequency of p53 mutation. Altogether, these results demonstrate that DTX3 binds to various p53 mutants in ovarian cancer.

DTX3 stabilizes mutant p53 by overcoming MDM2 inhibition

Although the DTX family proteins possess E3 ubiquitin ligase activity via a conserved RING finger domain, little is known about the substrates of DTX3. Thus, we tested if DTX3 mediates mtp53 ubiquitination by *in vivo* ubiquitination assay. As illustrated in Figure 2A, the ubiquitination of mtp53-R273H was indeed enhanced by ectopic expression of DTX3. The effect was specific to DTX3 overexpression and not due to artifact, because the IB for inputs indicated appropriate loading of each sample (Fig. 2A). Additionally, we sought to determine which lysine(s) of ubiquitin might be required for DTX3-mediated mtp53 ubiquitination. To this end, we performed a set of *in vivo* ubiquitination assays using plasmids encoding wild-type or lysine-mutant ubiquitins, and found that all the lysines except for the lysine K6 are responsible for mtp53 ubiquitination (Fig. 2B, C). The K11- and K48-linked ubiquitin chains trigger degradation more frequently, while K27, K29, K33, and K63 are usually associated with functional regulation of the substrate proteins.[37] Thus, our results suggested that mtp53 may be modified with multiple chain types at the same time, or with a mixed ubiquitin chain, which would be tempting to investigate in the future. Interestingly, stable overexpression of DTX3 in ES-2 cells dramatically increased the level of mtp53-S241F (Fig. 2D), while knockdown of DTX3 by two independent siRNAs reduced mtp53-S241F expression (Fig. 2E). Importantly, the reduction of mtp53 level upon DTX3 siRNAs could be markedly restored when cancer cells were treated with the proteasome inhibitor MG132 (Fig. 2F), suggesting that DTX3 may stabilize mtp53 protein by blocking its proteasomal degradation. Finally, we sought

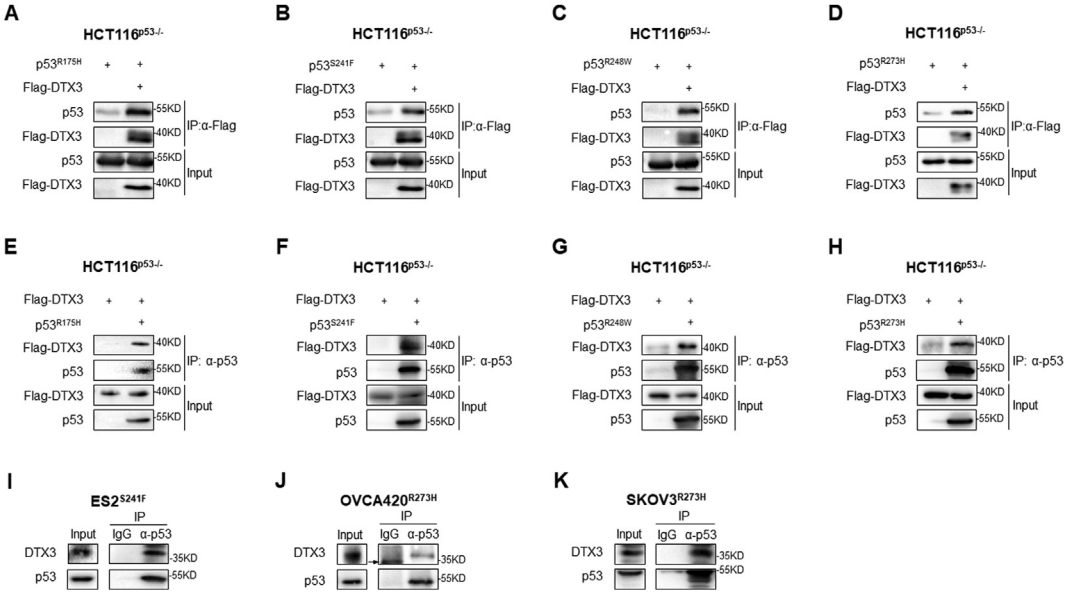

Figure 1 DTX3 physically interacts with mutant p53. **(A—H)** Exogenous DTX3 binds to exogenous mtp53 proteins by reciprocal co-IP assays. HCT116$^{p53-/-}$ cells were transfected with combinations of plasmids encoding Flag-DTX3 and different mtp53s, including R175H, S241F, R248W, and R273H, as indicated. The co-IP assays were performed using the anti-Flag (A—D) or anti-p53 antibody (E—H). **(I—K)** Endogenous interactions between DTX3 and mtp53 in the ovarian cancer cell lines, ES-2 (mtp53-S241F), OVCA420 (mtp53-R273H), and SKOV3 stably expressing mtp53-R273H. The co-IP assays were conducted by the anti-p53 antibody followed by IB analysis using antibodies as indicated. The arrow in the panel J indicates a non-specific band.

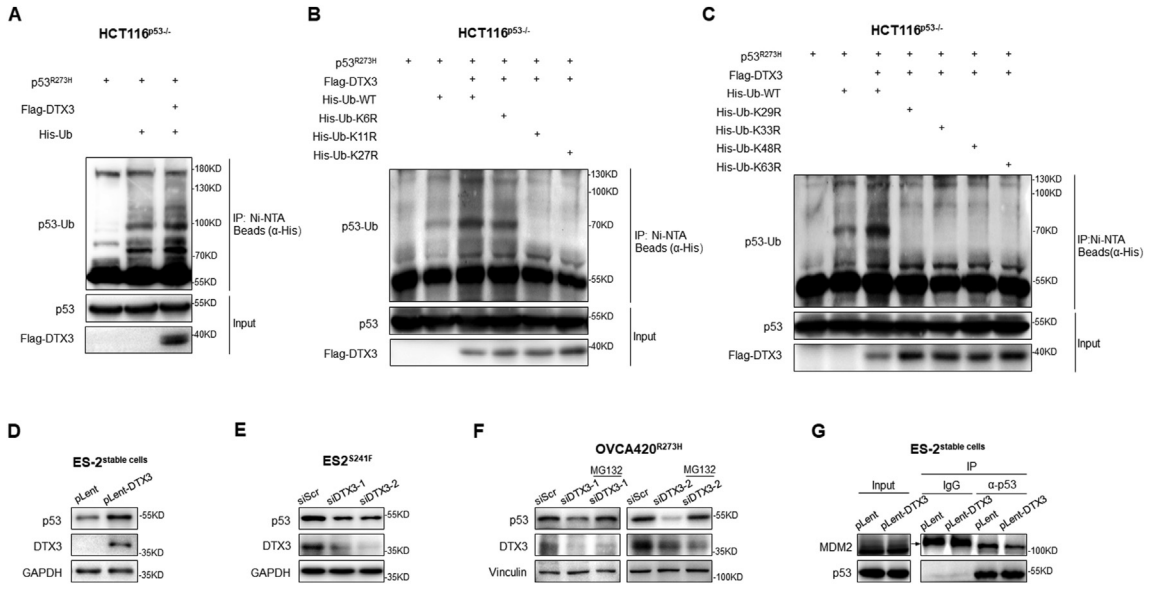

Figure 2 DTX3 stabilizes mutant p53 by reducing MDM2-p53 binding. **(A)** DTX3 induces ubiquitination of mtp53. HCT116$^{p53-/-}$ cells were transfected with combinations of plasmids as indicated, and treated with MG132 (20 μM) for 6 h before harvested for *in vivo* ubiquitination assay. **(B, C)** The lysines K11, K27, K29, K33, K48, and K63 of the ubiquitin are important for DTX3-induced mtp53 ubiquitination. The same experiment was performed as that in the panel A, except that the lysine-mutant ubiquitin plasmids were used. **(D)** Overexpression of DTX3 increases mtp53 protein level. ES-2 cells infected with control or DTX3-containing lentivirus were subjected to IB analysis using antibodies as indicated. **(E)** Knockdown of DTX3 reduces mtp53 protein level. ES-2 cells were transfected with siRNAs against DTX3 followed by IB analysis using antibodies as indicated. **(F)** DTX3 siRNA-mediated reduction of mtp53 level is restored by the proteasome inhibitor MG132. OVCA420 cells were transfected with siRNAs against DTX3 and treated with MG132 for 6 h before harvest for IB analysis using antibodies as indicated. **(G)** Ectopic DTX3 interferes with the MDM2-mtp53 interaction. ES-2 cells stably expressing the empty vector or DTX3 were subjected to co-IP-IB analysis using antibodies as indicated. The arrows indicate non-specific bands.

to explore the molecular basis of DTX3-induced mtp53 stabilization. Since poly-ubiquitination has been found to play a critical role in modulating protein—protein interactions,[37] we tested if DTX3 perturbs the interaction between mtp53 and MDM2, a master E3 ubiquitin ligase that promotes proteasomal degradation of mtp53 [38,39]. MDM2 could be co-immunoprecipitated with mtp53-S241F by an anti-p53 antibody in ES-2 cells, while their interaction was impaired when DTX3 was stably overexpressed in these cells (Fig. 2G). Therefore, these results demonstrate that DTX3 induces mtp53 stabilization by preventing MDM2 binding to mtp53.

DTX3 activates mutant p53 target gene expression

Although p53 mutants are despoiled of their ability to associate directly with DNA elements, they can still activate gene transcription by interacting with and potentiating other transcription factors or co-factors.[14] Thus, we determined if ectopic DTX3 induces mtp53 target gene expression through stabilization of mtp53. The lentivirus or plasmid encoding DTX3 was introduced into ES-2, OVCA420, and SKOV3-R273H cells followed by RT-qPCR analysis. As a consequence, overexpression of DTX3 significantly elevated the expression of mtp53 target genes (Fig. 3A—C). Conversely, ablation of DTX3 dramatically repressed mtp53 target gene expression (Fig. 3D). These target genes are associated with a wide range of cellular processes. For instance, IGFBP3, MAP2K3, MYC, NFKB2, CXCL1, and BCL2L1 are required for cell survival and proliferation. BUB1, CCNB1, CCNA2, NCAPH, and CENPA are critical for cell cycle progression. PXN, ITGA6, and MMP13 are diver genes of EMT and metastasis. RANGAP1 and ARHGDIA are responsible for energetic metabolism. We further validated that the effect of DTX3 is due to regulation of mtp53, because depleting mtp53 by siRNA completely restored the gene expression levels induced by DTX3 (Fig. 3E).

Next, we explored if DTX3-induced activation of mtp53 could be reflected in ovarian carcinomas. In agreement with the cell-base experiments, we revealed a positive correlation between the expression of DTX3 and some of the mtp53 target genes by mining the TCGA database (Fig. 3F—J). These correlations also suggest that DTX3 might promote ovarian cancer cell cycle progression, proliferation, survival, migration, and lipid metabolism. Collectively, these results demonstrate that DTX3 upregulates gene expression by activating mtp53, which might lead to the tumorigenic outcome.

DTX3 promotes ovarian cancer cell proliferation and invasion dependent on mutant p53

Since DTX3 modulates the expression of a broad spectrum of mtp53 target genes, we determined if this could lead to its biological function in ovarian cancer development. A set of CCK-8 cell viability assays was performed using mtp53-

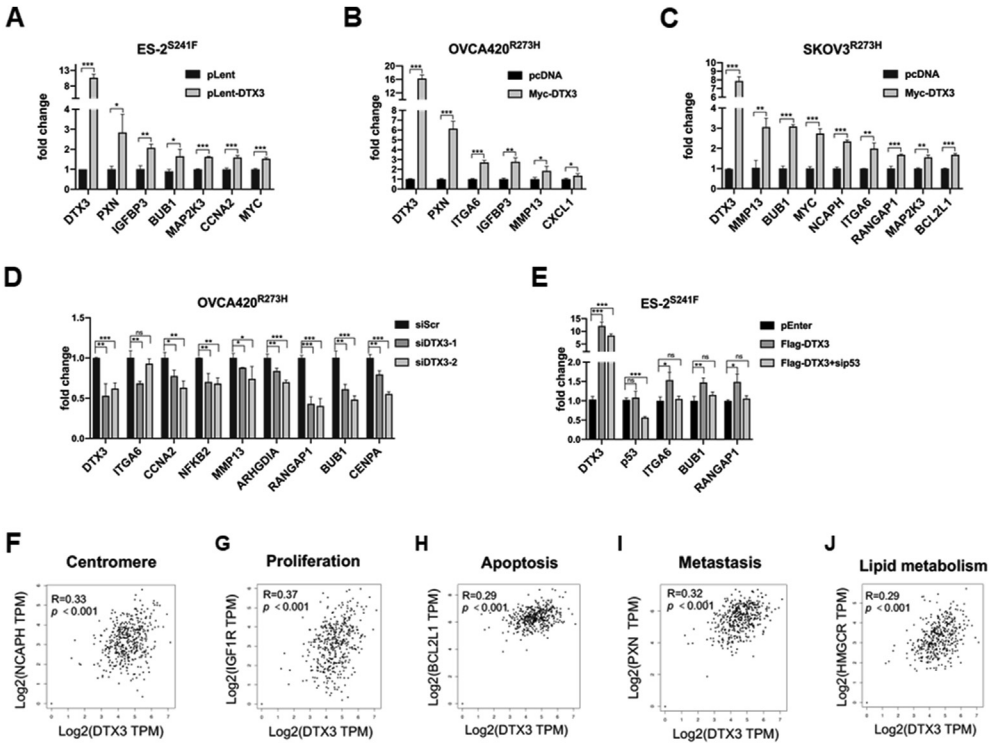

Figure 3 DTX3 induces mutant p53 target gene expression in ovarian cancer. **(A—C)** Overexpression of DTX3 upregulates mtp53 target gene expression. ES-2 cells stably expressing the empty vector or DTX3 were subjected to RT-qPCR analysis (A). OVCA420 cells were transfected with the empty vector or the DTX3-encoding construct followed by RT-qPCR analysis (B). mtp53-R273H-stably expressing SKOV3 cells were transfected with the empty vector or the DTX3-encoding construct followed by RT-qPCR analysis (C). **(D)** Knockdown of DTX3 reduces mtp53 target gene expression. OVCA420 cells were transfected with control or DTX3 siRNAs followed by RT-qPCR analysis. **(E)** Knockdown of mtp53 restores DTX3-induced gene expression. ES-2 cells were transfected with combinations of plasmids and siRNAs as indicated followed by RT-qPCR analysis. All the experiments were performed in biological triplicate. **(F—J)** The positive correlation between expression of DTX3 and mtp53 target genes involved in various biological outcomes, such as cell cycle (F), proliferation (G), apoptosis resistance (H), metastasis (I), and lipid metabolism (J) in ovarian carcinomas.

containing ES-2 and OVCA420 cells. Ectopic DTX3 significantly accelerated the proliferation of ES-2 and OVCA420 cells (Fig. 4A, B), while ablation of DTX3 by two independent siRNAs retarded cell proliferation (Fig. 4C). Also, the flow cytometry analysis showed that ectopic DTX3 significantly suppresses apoptosis of ES-2 cells (Fig. 4D). These observations were in concordance with the former results showing that DTX3 activates a number of proliferation-associated genes (Fig. 3). In addition, the transwell cell invasion assay was conducted to test if DTX3 regulates cancer cell metastasis, as it also promoted the expression of metastasis-driving genes (Fig. 3). As shown in Figure 4E, ectopic DTX3 drastically boosted the invasive potential of OVCA420 cells. Conversely, knockdown of DTX3 markedly inhibited ES-2 cell invasion (Fig. 4F). Therefore, these results demonstrate that DTX3 acts as an oncogenic protein in the development of ovarian cancer.

Next, we wondered if mtp53 is required for DTX3-stimulated cancer cell proliferation and invasion. Ectopic DTX3 significantly motivated OVCA420 cell proliferation, while depletion of mtp53 impaired the effect of DTX3 on cell growth (Fig. 5A). This observation was also validated using two isogenic cell lines, SKOV3 and SKOV3-R273H. Ectopic DTX3 promoted SKOV3-R273H cell proliferation, but had a trivial effect on proliferation of p53-null SKOV3 cells (Fig. 5B). Consistently, overexpression of DTX3 triggered invasion of ES-2 and OVCA420 cells, but the effect was abolished by depletion of mtp53 in these cells (Fig. 5C, D). Altogether, these findings demonstrate that DTX3 motivates ovarian cancer growth and metastasis through activation of mtp53.

DTX3 drives ovarian tumor growth *in vivo* and is associated with unfavorable prognosis

To determine the biological function of DTX3 during ovarian carcinogenesis, we established the ovarian tumor xenograft model by subcutaneously inoculating ES-2 cells stably overexpressing DTX3 or the empty vector into nude mice. In agreement with the cell-based results, overexpression of DTX3 dramatically accelerated ovarian tumor growth *in vivo* compared with the control group as determined by the tumor volumes (Fig. 6A). Accordingly, the tumor weight and mass markedly increased because of overexpression of DTX3 (Fig. 6B, C). Next, we inquired if the increased growth of ovarian tumors is associated with activation of the mtp53 signaling pathway. Thus, the dissected tumors were further

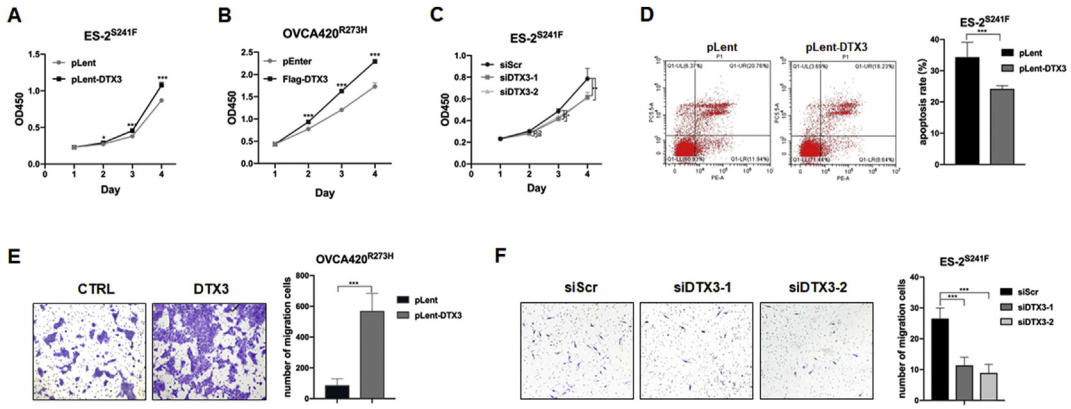

Figure 4 DTX3 promotes proliferation and invasion of ovarian cancer cells. **(A, B)** Overexpression of DTX3 prompts ovarian cancer cell proliferation. ES-2 cells stably expressing the empty vector or DTX3 were subjected to the CCK-8 cell viability assay for four days (A). OVCA420 cells were transfected with the empty vector or the DTX3-encoding plasmid followed by the CCK-8 cell viability assay at the indicated time points (B). **(C)** Knockdown of DTX3 suppresses ovarian cancer cell proliferation. ES-2 cells were transfected with control or DTX3 siRNAs followed by the CCK-8 cell viability assay. **(D)** Overexpression of DTX3 inhibits ovarian cancer cell apoptosis. ES-2 cells stably expressing the empty vector or DTX3 were subjected to the Annexin V staining and flow cytometry analysis. Quantification of the apoptotic cells is shown in the right panel. **(E)** Overexpression of DTX3 drives ovarian cancer cell invasion. OVCA420 cells stably expressing the empty vector or DTX3 were subjected to the transwell invasion assay. Quantification of the migrating cells is shown in the right panel. **(F)** Knockdown of DTX3 suppresses ovarian cancer cell invasion. ES-2 cells were transfected with control or DTX3 siRNAs for 24 h followed by the transwell cell invasion assay. Quantification of the migrating cells is shown in the right panel. All the experiments were performed in biological triplicate.

analyzed for the expression of mtp53 target genes by RT-qPCR. In line with the former results (Fig. 3A—C), a multitude of mtp53 target genes were significantly upregulated in DTX3-overexpressing tumors (Fig. 6D). To translate our findings to clinical significance, we examined the expression of DTX3 in normal and cancerous ovarian tissues through the Oncomine database (http://www.oncomine.org/). DTX3 was found to be amplified and overexpressed in ovarian carcinomas compared with normal tissues (Fig. 6E, F). The Kaplan—Meier survival analysis (kmplot.com) further unveiled that the higher level of DTX3 is associated with worse prognosis of ovarian cancer (Fig. 6G). Taken together, these findings demonstrate that high level of DTX3 is significantly associated with the progression of ovarian cancer in xenograft-bearing mice and human patients.

Discussion

Ovarian cancer sustains the highest frequency of *TP53* gene mutation across all human cancers. mtp53 has been shown to play an essential role in predisposition, progression, and therapeutic resistance of ovarian carcinomas. Thoroughly elucidating the regulation of mtp53 activity is of critical importance for better understanding of the mechanism and treatment of this cancer. In this study, we uncovered that the E3 ubiquitin ligase DTX3 interacts with and induces ubiquitination of mtp53. Interestingly, high level of DTX3 enhanced stabilization of mtp53 by preventing MDM2's association and inhibition of the latter, consequently leading to mtp53-dependent activation of proliferation- and metastasis-associated gene expression (Fig. 7). Hence, our study as presented above has demonstrated an oncogenic DTX3-mtp53 axis in ovarian cancer.

The DTX family of E3 ligases contains a highly conserved C-terminal RING finger domain and has been found to mediate ubiquitination and degradation of various substrates.[26] DTX1, DTX2, and DTX4 are able to modulate the NOTCH signaling by physically interacting with the intracellular domain of NOTCH via the N-terminal basic domains.[26,27] Interestingly, DTX1 was identified as a target gene that is transcriptionally regulated by NOTCH1, as thus forming a feedback regulatory circuit.[40] Additionally, DTX1 was found to be involved in lymphocyte development by negatively regulating NOTCH1 and, for example, MEKK1.[30,41] However, DTX3 is an underappreciated E3 ubiquitin ligase whose substrates and biological function remain incompletely understood. Previous studies indicated that DTX3 may be incapable of interacting with NOTCH, as it lacks the critical motif responsible for the binding. Interestingly, it was recently reported that DTX3 can bind to and prompt degradation of NOTCH2 in esophageal carcinoma cells, resulting in the inhibition of cancer cell growth and proliferation.[42] This might be a cancer context-specific interaction between DTX3 and NOTCH, which advanced our knowledge of DTX3's function in esophageal cancer. Our findings, by contrast, revealed that DTX3 acts as a tumor promoter by interacting with and bolstering mtp53 activity in ovarian cancer. Ectopic DTX3 boosted proliferation and suppressed apoptosis of ovarian cancer cells (Fig. 4A, B, D), while knockdown of DTX3 retarded ovarian cancer cell growth (Fig. 4C). Also, overexpression of DTX3 promoted, whereas ablation of DTX3 inhibited, ovarian cancer cell invasion (Fig. 4E, F). Consistently, the tumor xenograft mouse model also showed that overexpression of DTX3 prompts ovarian tumor growth *in vivo* (Fig. 6A—C). Further supporting this notion was that DTX3 is amplified and overexpressed in ovarian carcinomas

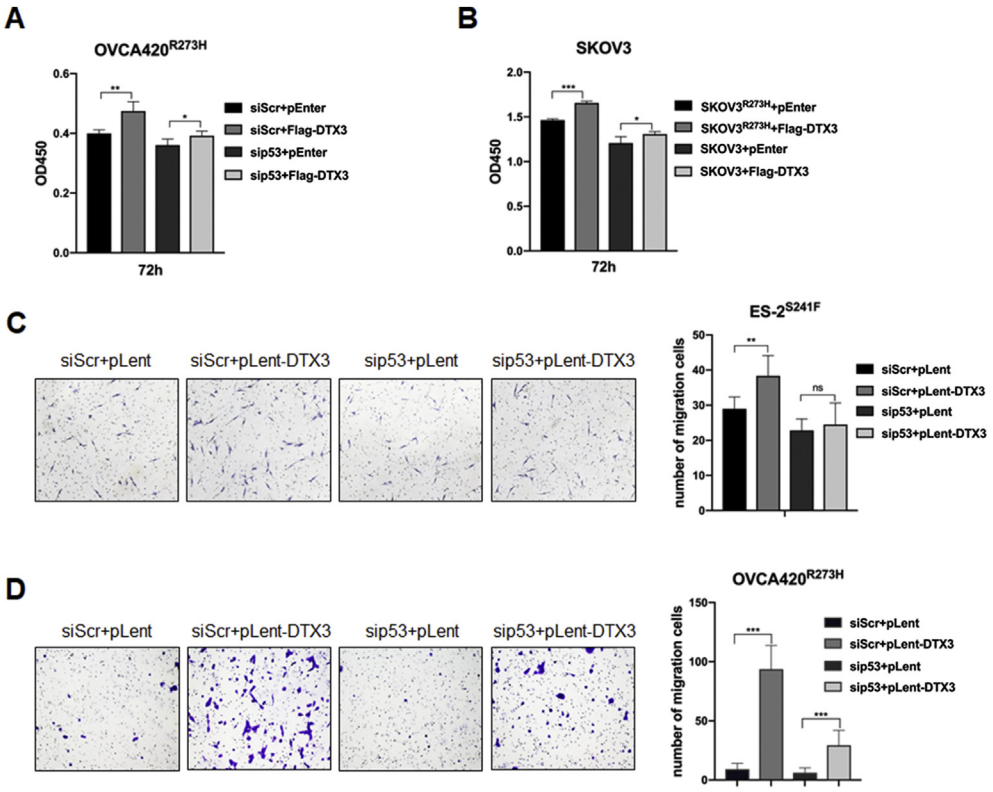

Figure 5 The pro-tumorigenic effect of DTX3 is dependent on mutant p53. **(A, B)** Depletion of mtp53 impairs DTX3-induced cell growth. OVCA420 cells were transfected with combinations of plasmids and siRNAs as indicated followed by the CCK-8 cell viability assay at 72 h post-transfection (A). SKOV3 cells stably-expressing the empty vector or mtp53-R273H were transfected with plasmids as indicated followed by the CCK-8 cell viability assay at 72 h post-transfection (B). **(C, D)** Depletion of mtp53 impairs DTX3-induced cell invasion. ES-2 (C) and OVCA420 (D) cells stably expressing the empty vector or DTX3 were transfected with siRNAs as indicated followed by the transwell cell invasion assay. Quantification of the migrating cells is shown in the right panels. All the experiments were performed in biological triplicate.

(Fig. 6E, F), which predicts poor prognosis (Fig. 6G). Therefore, these findings convincingly demonstrate that DTX3 promotes ovarian carcinoma cell growth and propagation as an oncogenic protein, and as thus is significantly associated with clinical outcomes of ovarian cancer.

Our study further elaborated that DTX3 exerts the pro-tumorigenic effect by enhancing mtp53 activity in ovarian carcinoma. First, we showed that ectopic DTX3 increases mtp53 level, while ablation of DTX3 reduces mtp53 expression (Fig. 2D, E). Also, overexpression of DTX3 significantly induced the expression of a broad range of mtp53 target genes that are responsible for diverse biological consequences, including cell cycle progression, apoptosis resistance, EMT and metastasis, and GTP metabolism (Fig. 3A—C). Remarkably, knockdown of mtp53 completely abated the induction of these target genes (Fig. 3E), indicating that DTX3 upregulates gene expression through mtp53. In addition, we ascertained that mtp53 activity is required for the oncogenic potential of DTX3, because depletion of mtp53 significantly impaired DTX3-induced ovarian cancer cell proliferation and motility (Fig. 5). Furthermore, the DTX3-overexpressing xenograft tumors exhibited higher levels of mtp53 target genes and accelerated growth (Fig. 6A—D). Together, these results explicitly decipher the role of the DTX3-mtp53 cascade in supporting ovarian cancer development.

In our attempt to elucidate the molecular basis of DTX3 regulation of mtp53, we found that DTX3 induces mtp53 poly-ubiquitination (Fig. 2A), but, surprisingly, ubiquitination of mtp53 led to stabilization of this protein (Fig. 2D—F). The ubiquitin molecule harbors seven lysines including K6, K11, K27, K29, K33, K48, and K63. Our results showed that six of these lysines, K11, K27, K29, K33, K48, and K63, are required for DTX3-inudced mtp53 ubiquitination (Fig. 2B, C). It is known that ubiquitin chains linked by different lysines can mediate diverse cellular functions or consequences of the substrates.[37] K11- and K48-linked ubiquitin chains often trigger proteolytic degradation of the substrates, while K29- and K63-linked chains mediate protein degradation less frequently. Actually, except for the K11- and K48-linked chains, all the other chain types were reported to be associated with the regulation of protein interactions, activity, or cellular localization.[37] We therefore speculated that DTX3-induced ubiquitination of mtp53 may alter the protein-binding capacity of the latter. Indeed, we attested that the interaction between MDM2

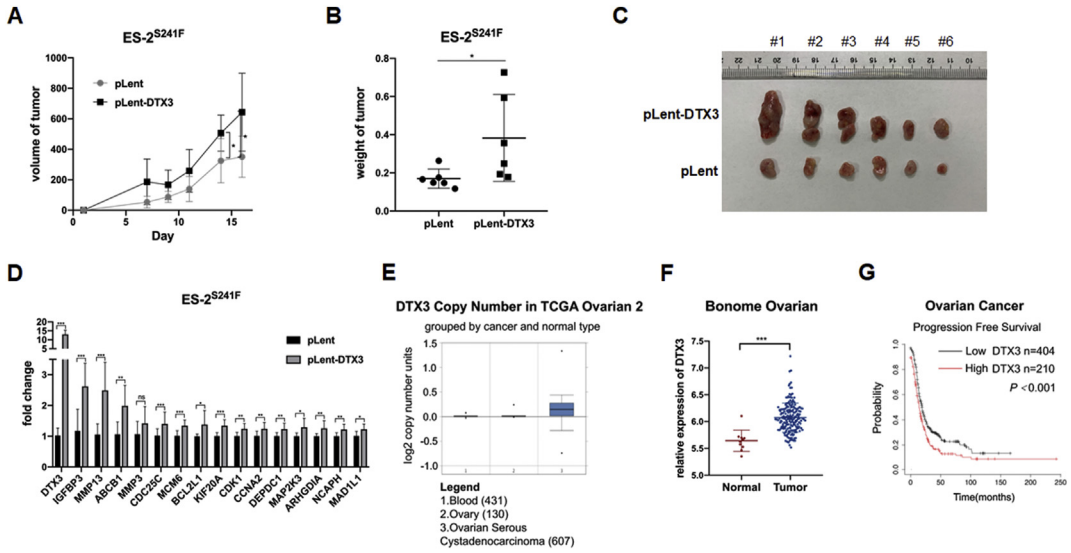

Figure 6 DTX3 fosters ovarian tumor growth *in vivo* and is negatively associated with prognosis. **(A)** Stable overexpression of DTX3 in ES-2 cells enlarges the xenograft tumor volume in average. **(B, C)** The dissected tumors show that stable overexpression of DTX3 increases tumor weight and mass. Data are represented as mean ± SD, $n = 6$. **(D)** DTX3 activates mtp53 target gene expression *in vivo*. The mRNA levels of mtp53 target genes in the dissected tumors were detected by RT-qPCR analysis. **(E)** The *DTX3* gene is amplified in ovarian cancer samples compared with blood and normal ovaries. **(F)** DTX3 mRNA is upregulated in cancerous versus normal ovarian tissues in the Bonome dataset.[43] **(G)** High level of DTX3 is associated with poor prognosis by the Kaplan–Meier survival analysis.

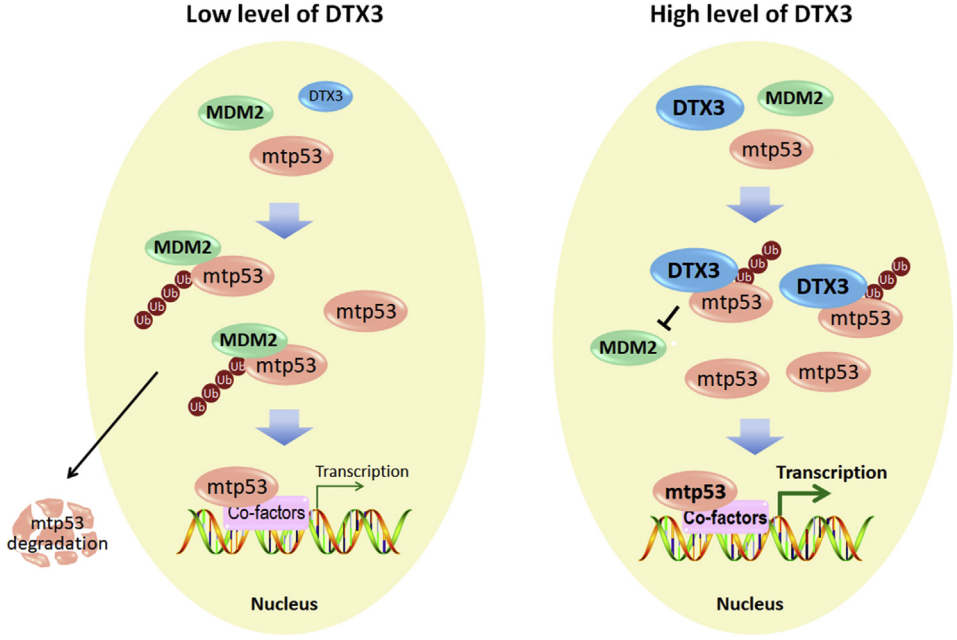

Figure 7 A schematic for the regulation of mutant p53 activity by DTX3 in ovarian cancer. In ovarian cancer cells with low levels of DTX3, MDM2, binds to and promotes proteasomal degradation of mtp53 (left panel). In ovarian cancer cells that sustain high levels of DTX3, DTX3 interacts with and mediates ubiquitination of mtp53, but instead of degrading p53, this action leads to inhibition of the MDM2-mtp53 interaction, consequently stabilizing mtp53 and inducing the expression of its target genes (right panel).

and mtp53 is impaired in the presence of DTX3 (Fig. 2G), which could be one of the reasons for DTX3-mediated mtp53 stabilization. Since mtp53 has been found to be degraded by multiple E3 ubiquitin ligases, such as PIRH2, COP1, CHIP, and TRIM71[36], perturbation of the interactions between these E3 ligases and mtp53 by DTX3 might be additional potential mechanisms for mtp53 stabilization. Another interesting question raised by this study is whether

DTX3-induced mtp53 ubiquitination plays a role in mtp53 interactions with other transcription factors or co-factors. Additionally, it remains unclear if DTX3 regulates wtp53 protein stability in other types of cancer whose *TP53* gene is less frequently mutated, because both wtp53 and mtp53 are often regulated by the same subset of E3 ubiquitin ligases in the context of different cancers.[36]

Conclusion

DTX3 is an understudied E3 ubiquitin ligase and its role in human cancers remains worthwhile investigating. Our study identified DTX3 as a novel binding protein of mtp53 in ovarian carcinoma. DTX3 mediated ubiquitination and stabilization of mtp53, probably via disruption of the MDM2-mtp53 interaction, leading to upregulation of a wide range of mtp53 target genes. Therefore, our findings demonstrate for the first time that DTX3 promotes ovarian cancer cell growth and propagation *in vitro* and *in vivo* by enhancing mtp53 activity.

Ethics approval and consent to participate

The animal protocols were in compliance with ethical regulations and approved by the Animal Welfare Committee of Shanghai Medical College at Fudan University.

Author contributions

S. W., Q. H. and J. L. conducted the experiments and analyzed the data; Q. H. designed part of the studies and instructed S. W. in several experiments. Y. C. provided part of critical experimental materials; X. W. and X. Z. designed the studies and analyzed the data; X. Z. composed the manuscript; H. L. assisted in study design and data analysis, and edited the manuscript.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2020.11.007.

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