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Original Article

DDX3 suppresses hepatocellular carcinoma progression through modulating the secretion and composition of exosome

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Abstract: Due to the lack of predictive biomarkers and the lack of conspicuous symptoms at the early stage, hepatocellular carcinoma (HCC) remains difficult to diagnose and treat effectively. During cancer development, exosomes secreted from tumor cells carry functional molecules to surrounding recipient cells, thereby participating in the regulation of cancer progression. DDX3, a DEAD-box RNA helicase, performs many important functions in several cellular processes and is therefore implicated as a tumor suppressor in HCC. However, whether DDX3 affects the secretion and cargo sorting of HCC exosomes remains obscure. In this study, our results revealed that reduced DDX3 expression in HCC cells promoted the release of exosomes and enhanced the expression of several exosome biogenesis-associated proteins, such as exosome markers (e.g., TSG101, Alix, and CD63) and Rab proteins (e.g., Rab5, Rab11, and Rab35). By double knockdown of the expression of DDX3 and these exosome biogenesis-related factors, we confirmed that DDX3 participated in the regulation of exosome secretion by modulating the expression of these cellular factors in HCC cells. In addition, exosomes derived from DDX3-knockdown HCC cells enhanced cancer stem cell properties, including self-renewal capability, migration, and drug resistance, in recipient HCC cells. Moreover, up-regulation of the exosome markers TSG101, Alix, and CD63 as well as down-regulation of tumor-suppressive miR-200b and miR-200c were observed in exosomes derived from DDX3-knockdown HCC cells, which may account for the enhanced hepatic cancer stemness of the recipient cells treated with DDX3-knockdown HCC cell-derived exosomes. Taken together, our findings provide a new molecular mechanism supporting the tumor-suppressor role of DDX3 in HCC, which may contribute to the development of new therapeutic strategies against HCC.

Keywords: Hepatocellular carcinoma, exosome, RNA helicase DDX3, Rab protein, miR-200b, miR-200c, cancer stem cell

Introduction

Liver cancer is the sixth most common cancer worldwide, and hepatocellular carcinoma (HCC) accounts for approximately 90% of cases of liver cancer [1-5]. A recent study reported that HCC is the third leading cause of cancer-related deaths globally, and the relative five-year survival rate is approximately 18% [6]. Due to the lack of obvious symptoms and predictive biomarkers, HCC is considered difficult to diagnose at the early stage and to treat properly. Therefore, specific markers for HCC are urgently needed to aid in the development of novel therapeutic strategies [4-7].

Exosomes are extracellular vesicles with particle diameters ranging from 30 to 150 nm [8-12]. They deliver diverse types of molecular messengers from donor cells to recipient cells, where they participate in many physiological functions [12-15], such as angiogenesis [16], autophagy [17], nerve protection [18], inhibition of viral infection [19], and immune modulation

[20]. Previous studies have indicated that during the development of various cancers, tumor cells alter the phenotype of surrounding recipient cells by releasing exosomes to promote cancer progression [8, 21-23]. Extensive studies have demonstrated that exosomes derived from HCC cells enhance the invasion and migration [24, 25], cell proliferation [26], drug resistance [27, 28], and metastatic ability [24, 29, 30] of recipient cells, thereby promoting the development of HCC [31-33]. As exosomes are heterogeneous in size and composition [10, 12, 13, 21, 34], investigating the secretion mechanism and the content of exosomes released from HCC cells will be conducive to designing more effective treatment strategies and developing predictive markers for the early diagnosis of HCC.

The biogenesis of exosomes is a complex process that originates from the endocytosis of the plasma membrane to form early endosomes, accompanied by the continuous inward budding and accumulation of intraluminal vesicles (ILVs) [9, 11, 14, 35]. Early endosomes gradually mature into late endosomes, also known as multivesicular bodies (MVBs), which ultimately fuse with the plasma membrane to secrete ILVs, referred to as exosomes [9, 13, 14, 35]. Many proteins and molecules regulate the process of exosome production, such as ESCRT (endosomal sorting complex required for transport) [35], Alix, tetraspanins (e.g., CD63, CD81, and CD9), lipids (ceramide and phosphatidic acid), and Rab proteins [11, 21, 22, 36-38]. In addition to participating in the formation or secretion of exosomes, the ESCRT components TSG101 [10, 39] and Alix [40] as well as the tetraspanin CD63 [41] are widely packaged into exosomes and are considered exosome markers. Rab proteins, small GTPases of the Ras superfamily, participate in intracellular vesicle transport and release by binding to their specific effectors, which are involved in vesicle formation, cargo selection, membrane tethering or docking, vesicle transport, and membrane fusion [9, 11, 12, 36, 42]. Rab proteins are classified based on their sequence similarity, and most Rab proteins have isoforms that are referred to as isoforms a, b, or c, if applicable [43]. Among the isoforms of a Rab protein, the function of isoform “a” is the most extensively studied. To date, the regulatory role of each Rab protein and its effector in the endocytic trafficking system has not been fully explored, but it seems that Rab5, Rab7, Rab11, Rab27, and Rab35 engage in the regulation of exosome biogenesis [11, 12, 21, 22]. In HCC, Rab5 [44], Rab11 [45], Rab27 [46, 47], and Rab35 [48] have also been shown to be involved in both the regulation of exosome formation or secretion and the progression of HCC. For example, Rab5a [49, 50] and Rab11a [51-53] are overexpressed in the tumor tissues of HCC patients or in HCC cell lines, and this overexpression is correlated with enhanced proliferation and invasion as well as migration abilities of HCC cells. Therefore, Rab5a and Rab11a are considered to play oncogenic roles in HCC. The expression of Rab27a in HCC tissue is significantly lower than that in normal liver tissue, and Rab27a knockdown enhances the migration, chemotaxis, invasion, and epithelial-mesenchymal transition (EMT) of HCC cells [47]. However, a study reported that Rab27a is overexpressed in tumor tissues of HCC patients and in HCC cell lines, so that this overexpression is positively correlated with poor prognosis [54]. Another study also indicated that Rab27a promotes cancer stemness and drug resistance in HCC cells [46]. Thus, the role of Rab27a in HCC is not yet fully understood. According to these evidences, endosomal trafficking and exosome secretion mechanisms are closely associated with the development and malignancy of different cancers [46, 51, 55, 56].

DDX3, a member of the DEAD-box RNA helicase family, has multiple functions in RNA metabolism, such as RNA unwinding [57], transcription [58-62], mRNA export [57, 63], translation [63-68], and microRNA biogenesis [69, 70]. Therefore, DDX3 is involved in the regulation of the cell cycle [71, 72], cell growth [73], mitosis [74], apoptosis [75, 76], stress response [77, 78], and innate immunity as well as virus infection [57, 72, 79-83]. Previous studies have indicated that DDX3 acts as a double-edged sword in different types of cancers [84, 85]. As an oncogenic protein, DDX3 is overexpressed in highly invasive breast cancer cell lines and promotes cell growth, proliferation, and EMT by inhibiting the transcriptional activity of E-cadherin [59]. DDX3 also binds to circCTNNB1, activates the transcription factor Yin Yang 1 (YY1), and initiates the β-catenin signaling pathway to enhance the cell growth and invasion of colorectal cancer, gastric cancer, and prostate cancer [86]. Several studies have

also indicated that DDX3 plays a tumor-suppressive role in liver cancer. Decreased expression of DDX3 is negatively correlated with tumor stage and results in poor prognosis in HCC patients [87]. DDX3 binds with Sp1 and enhances the expression of $p21^{waf1/cip1}$, which inhibits tumor growth of HCC [58]. Moreover, DDX3 prevents DNA methyltransferase 3A (DNMT3A) from binding to the promoter region of miR-200b, miR-200c, miR-122, and miR-145, leading to the transcriptional activation of these tumor-suppressive microRNAs and inhibiting stem cell properties, including the self-renewal ability, drug resistance, migration, and EMT of HCC cells [87]. In addition, DDX3 regulates the expression of damage DNA binding protein 2 (DDB) and *xeroderma pigmentosum* complementation group A (XPA) to maintain genome stability. When DDX3 function is lost, unrepaired DNA damage and replication stress accumulate, which contributes to the increased incidence of HCC development in a mouse model [88]. However, whether DDX3 participates in the regulation of secretion and composition of HCC exosomes is unclear.

In this study, we found that the expression of DDX3 is negatively correlated with the number of exosomes released and the expression of exosome biogenesis-associated proteins in HCC cells, such as several exosome markers (TSG101, Alix, and CD63) and Rab proteins (Rab5, Rab11, and Rab35). We confirmed that DDX3 inhibits exosome secretion by regulating the expression of these exosome biogenesis-related factors. In addition, cancer stem cell properties, including self-renewal capability, migration, and drug resistance, were enhanced when HCC cells were treated with exosomes derived from DDX3-knockdown HCC cells. As the levels of several exosome biogenesis-related proteins and tumor-suppressive microRNAs were altered in DDX3-knockdown HCC cell-derived exosomes, DDX3 may possess the ability to affect the composition of HCC exosomes. Collectively, these findings provide new evidence for further strengthening the tumor-suppressive role of DDX3 in HCC.

Materials and methods

Plasmids

For short hairpin RNA (shRNA) lentivirus production, the plasmids pLKO.1-puro-based shRNA vectors, pLKO_TRC005-puro-based shRNA vectors, pCMV-ΔR8.74psPAX2 (plasmid that expresses pol, gag and rev) and pMD2.G (plasmid that expresses vesicular stomatitis virus glycoprotein) were purchased from the National RNAi Core Facility, Academia Sinica, Taiwan. The clone number of each shRNA plasmid was as follows: shLuc (TRCN00000722-46), shDDX3 #2 (TRCN0000000002), shDDX3 #3 (TRCN0000000003), shTSG101 (TRCN00-00007564), shAlix (TRCN0000029394), shCD63 (TRCN0000273631), shRab5a (TRCN-0000380466), shRab11a (TRCN00003812-43), and shRab35 (TRCN0000380335). The sequences of each shRNA targeting the coding sequence of a specific gene are listed in Table S1. The plasmids pcDNA3-SRα/Flag and pcDNA3-SRα/Flag-DDX3 were constructed as described previously [58].

Antibodies

The following antibodies were used in this study: anti-DDX3 (sc-81247, Santa Cruz Biotechnology, Dallas, TX, USA), anti-TSG101 (sc-7964, Santa Cruz Biotechnology), anti-Alix (#2171, Cell Signaling Technology, Danvers, MA, USA), anti-CD63 (sc-5275, Santa Cruz Biotechnology), anti-Hsc70 (sc-7298, Santa Cruz Biotechnology), anti-Hsp90 (#4877, Cell Signaling Technology), anti-Calnexin (#2679, Cell Signaling Technology), anti-Rab5a (ab-18211, Abcam, Cambridge, UK), anti-Rab5b (ab72907, Abcam), anti-Rab7a (#9367, Cell Signaling Technology), anti-Rab7b (ab193360, Abcam), anti-Rab11a (#2413, Cell Signaling Technology), anti-Rab11b (#2414, Cell Signaling Technology), anti-Rab27a (sc-74586, Santa Cruz Biotechnology), anti-Rab35 (#11329-2-AP, ProteinTech Group Inc, Chicago, IL, USA), anti-β-Actin (#A5441, Sigma-Aldrich, St. Louis, MO, USA), and anti-GAPDH (#G8795, Sigma-Aldrich).

Cell culture and transfection

HepG2 and HuH-7 cells were cultured in Minimum Essential Medium (MEM) (#SH30008.02, Cytiva, Marlborough, MA, USA) and Dulbecco's modified Eagle's medium (DMEM) (#SH30002.02, Cytiva), respectively, supplemented with 10% heat-inactivated fetal bovine serum (#10438-026, Gibco, Grand Island, NY, USA), 3 mg/ml L-glutamine, 1% nonessential amino acids, 100 unit/ml penicillin, and 100

μg/ml streptomycin. HEK293FT cells were cultured in DMEM (#SH30003.02, Cytiva) supplemented with 10% inactivated fetal bovine serum (#10437-028, Gibco), 0.3 mg/ml L-glutamine, 1% nonessential amino acids, 100 unit/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured in a humidified incubator at 37°C with 5% CO_2 (Forma 301, Thermo Fisher Scientific, Marietta, OH, USA). HepG2 cells were transfected with plasmid pcDNA3-SRα/Flag or pcDNA3-SRα/Flag-DDX3 using Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen). Cells were incubated with a mixture of Lipofectamine 2000 reagent and plasmid for 12 h, washed once with PBS, and incubated in complete medium for 24 h.

Lentivirus production and establishment of target gene-knockdown HCC cells

Lentivirus production in HEK293FT cells was carried out according to the protocol suggested by the RNAi Core Facility, Academia Sinica, Taiwan (http://rnai.genmed.sinica.edu.tw/webContent/web/protocols). HepG2 cells were infected with lentivirus in culture medium containing 10 μg/ml polybrene (#9268, Sigma-Aldrich) for 24 h and selected with 2 μg/ml puromycin (#P8833, Sigma-Aldrich) for at least 3 days. Target gene-knockdown HuH-7 cells were established with similar procedures to those described above, except that lentivirus-infected HuH-7 cells were selected with 1.5 μg/ml puromycin.

Exosome isolation

Prior to conditioned medium collection, the cell culture medium was replaced with serum-free medium for 24 h. The cell number was counted as the conditioned medium was collected. The conditioned medium was centrifuged at 2,000×g for 20 min at 4°C, and the supernatant was filtered through 0.45 μm filters to remove microvesicles, apoptotic bodies, and cell debris. To isolate exosomes by ultracentrifugation, the conditioned medium was ultracentrifuged at 10,000×g for 30 min at 4°C and then at 100,000×g for 2 h at 4°C using an SW28 rotor in an Optima XE-90 Ultracentrifuge (Beckman Coulter, Brea, CA, USA). The pellet was resuspended in PBS and further ultracentrifuged at 100,000×g for 70 min at 4°C using an SW60 rotor. Exosomes were also purified with Total Exosome Isolation Reagent (#4478359, Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

Transmission electron microscopy (TEM)

Exosome samples were prepared by ultracentrifugation as described above. The Formvar/carbon grid was placed in an EMITECH K100X Glow Discharge System (Emitech Ltd., Chelmsford, UK) at 25 mA for 30 sec. Then, 5 μl of exosome sample was dropped on the grid and incubated for 1 min, and the solution was removed with filter paper. For negative staining, 5 μl of 1% uranyl acetate was dropped on the sample grid and incubated for 30 sec, excess solution was removed with filter paper, and the sample grid was completely dried. The TEM images were acquired using an FEI Tecnai G2 F20 S-TWIN transmission electron microscope at 120 kV.

Nanoparticle tracking analysis (NTA)

Exosome samples were prepared by ultracentrifugation as described above and diluted with PBS at a concentration range from 1×10^6 to 1×10^9 particles/ml before nanoparticle tracking analysis (NanoSight NS300, Malvern Panalytical, Malvern, UK) was carried out. The size and concentration of the nanoparticles were analyzed at a constant flow rate of 70 μl/min at 25°C.

Quantitative polymerase chain reaction (qPCR)

The extraction of total cellular RNA, preparation of cDNA, and analysis of mRNA or miRNA expression by qPCR were performed as described previously [87]. Total RNA in exosomes was extracted by using a Total Exosome RNA and Protein Isolation Kit (#4478545, Invitrogen) according to the manufacturer's instructions. The primers used in qPCR analysis are listed in Table S2.

Sphere formation assay

For HepG2 cells, parental cells (1×10^6 cells/well in a 12-well plate) were treated with exosomes derived from 1.5×10^6 control or DDX3-knockdown HepG2 cells for 24 h. For HuH-7 cells, parental cells (4×10^5 cells plated in 6-well plates) were treated with exosomes derived from 8×10^5 control or DDX3-knockdown HepG2 cells. Parental cells treated with serum-free

medium were used as a mock control. Treated cells were subjected to the sphere formation assay as previously described [89]. Briefly, single-cell suspensions of each group of exosome-treated HepG2 or HuH-7 cells were cultured in Ultra-Low Attachment 6-well plates (#3471, Corning, Tewksbury, MA, USA). After 7 to 10 days of incubation, the numbers of formed spheres were counted, and images were captured using an inverted microscope WI-400 with a UC-850 camera (WHITED, Washington, DC, USA).

Transwell migration assay

Parental HepG2 and HuH-7 cells were treated with serum-free medium and DDX3-knockdown HepG2 cell-derived exosomes as described above. Cells resuspended in serum-free MEM were added into the upper chamber of the transwell insert (#3464, Corning Costar), and MEM containing 10% HI-FBS was loaded into the lower chamber. After 24 or 72 h of incubation for HuH-7 or HepG2 cells, respectively, cells were fixed with methanol-acetone (1:1) solution and stained with Giemsa (Sigma-Aldrich) or crystal violet (#1.15940.0025, MERCK, Rahway, NJ, USA). Then, the transwell insert was washed, the unmigrated cells were removed by cotton swabs, and the migrated cells were visualized and counted in six randomly chosen fields at a magnification of 40× by inverted microscope WI-400 with UC-850 camera (WHITED).

MTS assay

Parental HepG2 and HuH-7 cells were treated with exosomes as mentioned above, seeded into 96-well plates, and treated with anti-cancer drugs doxorubicin (0, 0.05, 0.1, 0.2, and 0.4 μg/ml) and 5-fluorouracil (0, 0.25, 0.5, 1, and 2 μg/ml) for 72 h. Cell viability was analyzed by cellular ability to transform 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, G3582, Promega, Madison, WI, USA) into red formazan, and the absorbance was determined at 490 nm.

Results

DDX3 inhibits exosome secretion in HCC cells

To investigate whether DDX3 affects the secretion of exosomes in HCC cells, HepG2 and HuH-7 cells were infected with lentivirus containing DDX3 shRNA to establish DDX3-knockdown HCC cells, and the knockdown efficiency was confirmed by western blotting (**Figure 1A**). Conditioned medium was collected from the control (shLuc) and DDX3-knockdown (shDDX3 #2 and shDDX3 #3) cells, and the exosomes were purified by ultracentrifugation. Isolated round-shaped particles of approximately 100 nm were observed under transmission electron microscopy (TEM) to identify the characteristics of exosomes (**Figure 1B**). Furthermore, nanoparticle tracking analysis (NTA) was performed to analyze the nanoparticle size distribution and the concentration of isolated exosomes (**Figure 1C**). The average diameter of HepG2 or HuH-7 cell-released exosomes was between 76.6 and 129.5 nm, which corresponded to the expected size of exosomes. The concentration of exosomes obtained from NTA was normalized to the sample volume as well as the secreted cell number to calculate the average number of released exosomes per cell. We found that decreased expression of DDX3 in HCC cells significantly increased the number of exosomes secreted by HepG2 and HuH-7 cells (**Figure 1D**). This finding indicated that DDX3 may inhibit exosome release from HCC cells. To further confirm whether DDX3 has an inhibitory effect on exosome secretion of HCC cells, a vector control and plasmid expressing Flag-DDX3 were transiently transfected into HepG2 cells (**Figure 2A**), and the conditioned medium was collected and subjected to exosome isolation. The size distribution and concentration of exosomes derived from DDX3-overexpressing HepG2 cells were identified using NTA as described above. Our results demonstrated that the particle size of exosomes was approximately 110 nm (**Figure 2B**), and the secretion of exosomes per cell was significantly reduced under DDX3 overexpression (**Figure 2C**). Therefore, our results revealed that DDX3 plays an inhibitory role in the exosome secretion of HCC cells.

DDX3 affects the expression levels of exosome biogenesis-associated factors in HCC cells

Exosomes are a type of extracellular vesicle with a double-layered phospholipid membrane, and the membrane often contains molecules such as tetraspanins (e.g., CD63, CD81, and CD9) and ESCRT components (e.g., TSG101 and Alix) [11, 12, 22]. These proteins are

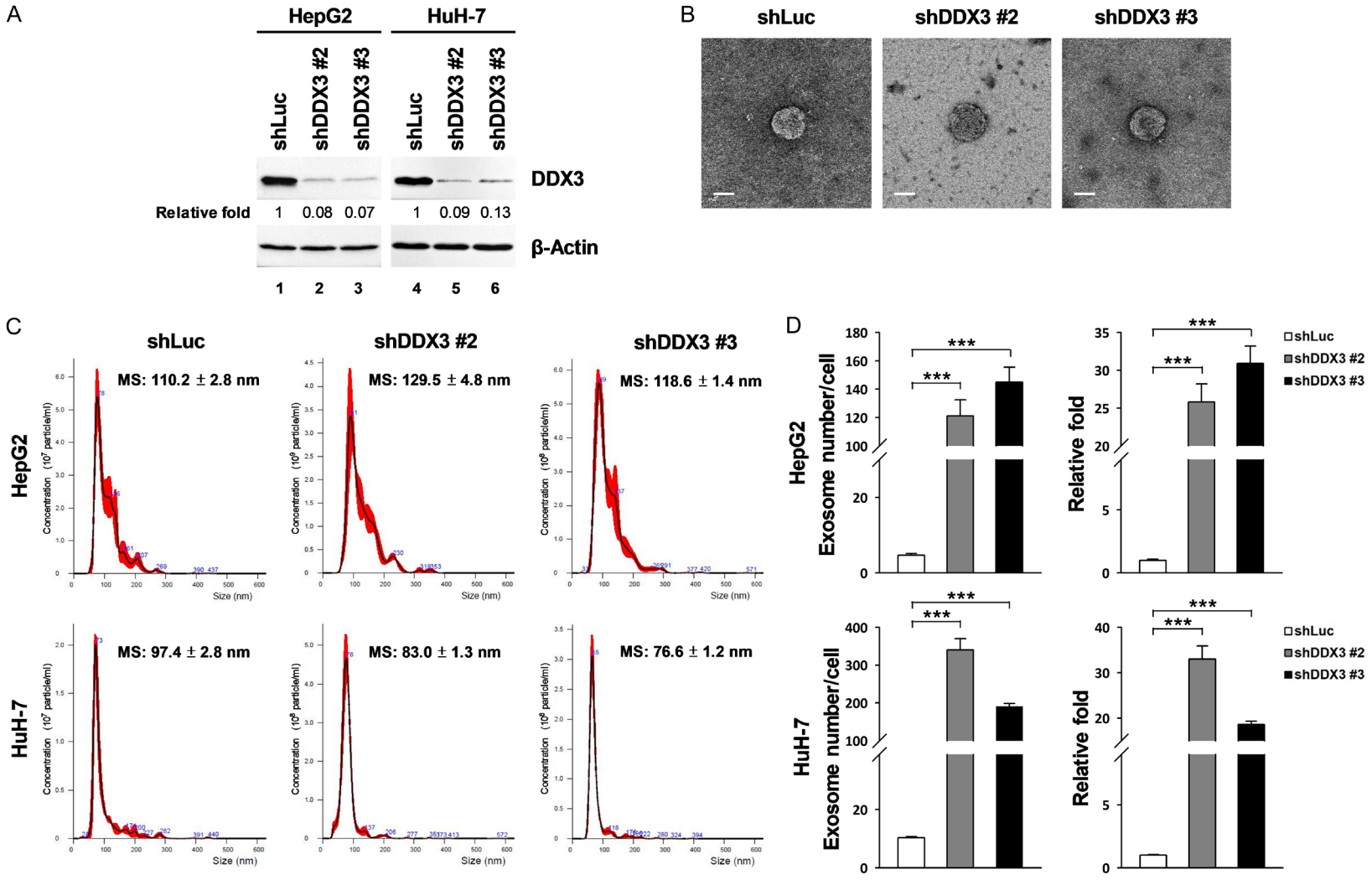

Figure 1. Decreased DDX3 expression enhances exosome secretion in HCC cells. A. The establishment of control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HCC cells. Cell lysates (50 μg) were analyzed by western blotting using anti-DDX3 and β-actin antibodies. The western blot intensities were quantified by using Lab Image software in ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA), which was calibrated to that of the loading control β-actin. The results were normalized to the expression level in control cells. B. Morphology and particle size of purified exosomes. The exosomes released from control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HepG2 cells were purified using ultracentrifugation and characterized by TEM. Scale bar represents 50 nm. C. The size distribution of purified exosomes. The exosomes secreted by control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HCC cells were isolated by ultracentrifugation and analyzed by nanoparticle tracking analysis (NTA). D. Knockdown of DDX3 promoted exosome secretion in HCC cells. The concentrations of exosomes from control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HCC cells were determined by using NTA. The number of exosome particles/cell was calculated according to

the following formula: (exosome concentration × sample volume)/(cell number as the conditioned medium was collected). The number of exosomes secreted per cell was transformed into the fold change relative to that of control cells. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (Systat Software Inc., Chicago, IL, USA) (***, P < 0.001). The data shown are from one representative experiment of three independent experiments. MS, mean size.

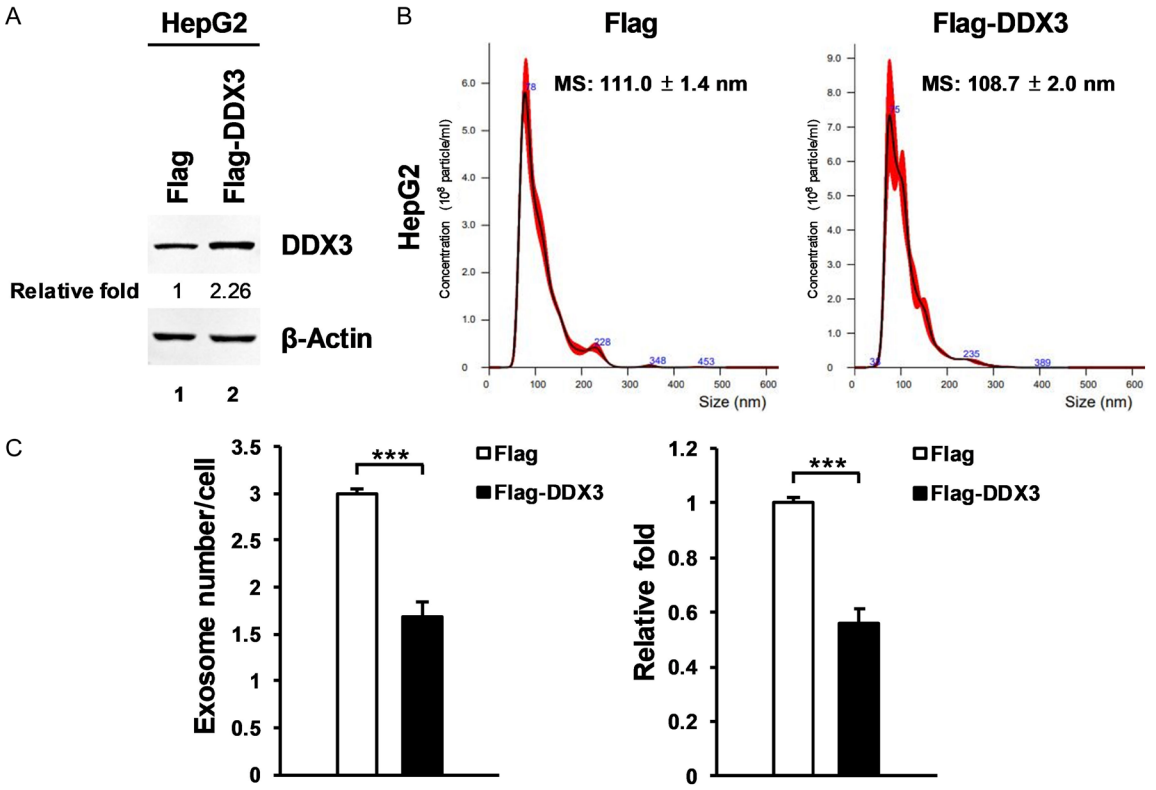

Figure 2. Overexpression of DDX3 suppresses exosome secretion of HepG2 cells. A. Overexpression of DDX3 in HepG2 cells. Plasmid pcDNA3-SRα/Flag (Flag) or pcDNA3-SRα/Flag-DDX3 (Flag-DDX3) was transiently transfected into HepG2 cells by Lipofectamine 2000 reagent. Cell lysates (25 μg) were analyzed by western blotting using anti-DDX3 and β-actin antibodies. Quantification and calibration of western blot intensities were performed using β-actin as the loading control. The results were then normalized relative to the expression level in control cells. B. The size distribution of purified exosomes. The exosomes released from vector control (Flag) or DDX3-overexpressing (Flag-DDX3) HepG2 cells were purified by ultracentrifugation and analyzed using NTA. C. Exosome secretion was inhibited in DDX3-overexpressing HepG2 cells. The concentrations of exosomes derived from vector control (Flag) and DDX3-overexpressing (Flag-DDX3) HepG2 cells were determined by NTA. The number of exosome particles/cell was calculated as described in the legend of **Figure 1D**. The number of exosomes secreted per cell was transformed into the fold change relative to that of vector control cells. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (***, P < 0.001). The presented data are from a single experiment that is representative of three independent experiments. MS, mean size.

involved in the regulation of exosome biogenesis and are widely recognized as exosome markers. To further verify the effect of DDX3 on exosome secretion of liver cancer cells, exosomes derived from DDX3-knockdown HepG2 or HuH-7 cells were isolated, and the expression level of exosome markers was detected by western blotting. As shown in **Figure 3A**, reduced expression of DDX3 in HepG2 or HuH-7 cells resulted in increased expression levels of TSG101, Alix, and CD63, while no significant changes were observed in the expression of Hsc70 and Hsp90 in cell lysates. In the exosomes released by these two DDX3-knockdown HCC cell lines, the expression levels of TSG101, Alix, CD63, Hsc70, Hsp90, and GAPDH were significantly increased. The endoplasmic reticulum (ER)-specific marker calnexin was also included in the western blotting analysis and was not observed in the isolated exo-

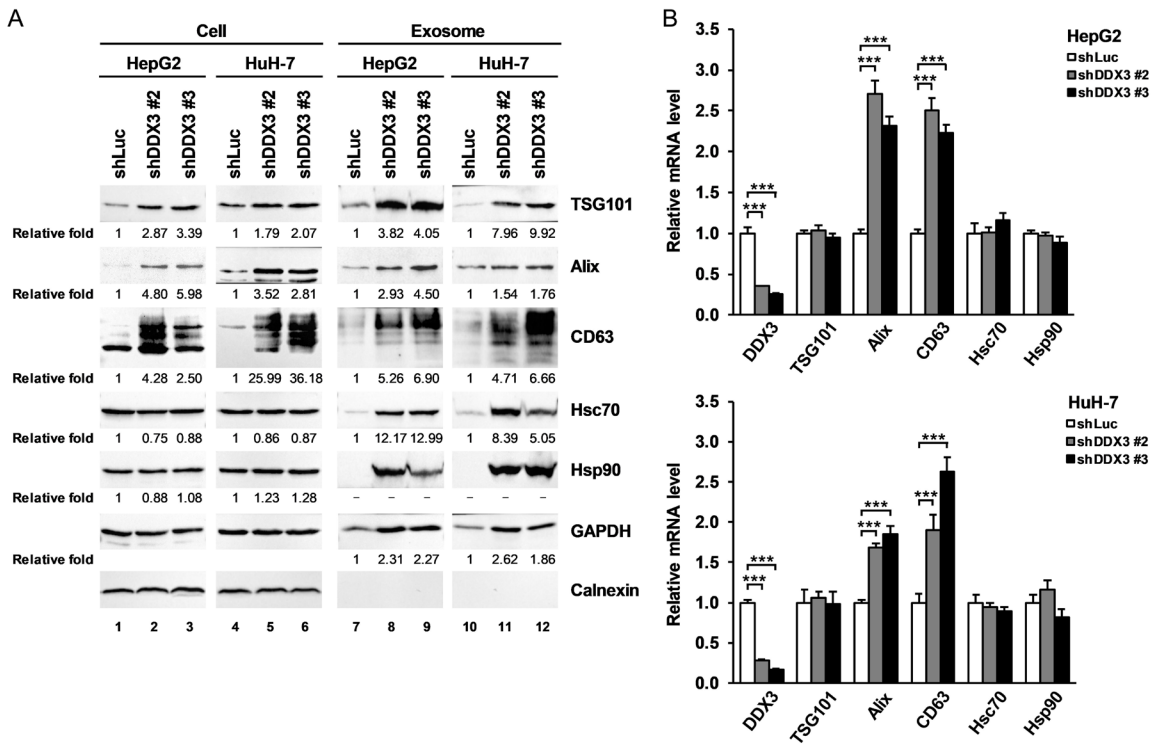

Figure 3. Reduced DDX3 leads to higher expression of TSG101, Alix, and CD63. A. The cellular and exosomal levels of several exosome markers were analyzed in HepG2 and HuH-7 cells. The exosomes released from control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HCC cells were purified with Total Exosome Isolation Reagent. Cell lysates (50 μg) and exosome proteins (150 μg) were analyzed by western blotting with antibodies against TSG101, Alix, CD63, Hsc70, Hsp90, GAPDH, and calnexin. The cellular level of proteins was quantified and calibrated to that of the loading control GAPDH, which was normalized to the expression level in control cells. The exosomal level of proteins was quantified and normalized to the level in control cells. B. The mRNA expression levels of Alix and CD63 were significantly increased in DDX3-knockdown HCC cells. The mRNA expression of DDX3, TSG101, Alix, CD63, Hsc70, Hsp90, and β-actin in control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HCC cells was analyzed by qPCR. The mRNA level of each gene was calibrated with that of β-actin. The fold change in each mRNA transcript in DDX3-knockdown cells was expressed relative to that in control cells. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (***, $P < 0.001$). One of three independent experiments is shown as a representative example of the data.

somes. These observations implied that DDX3 knockdown may promote the release of exosomes by up-regulating the expression of exosome markers in HCC cells.

To evaluate whether DDX3 regulates the expression of exosome markers at the transcriptional level, qPCR was used to analyze the mRNA levels of these exosome markers in DDX3-knockdown HCC cells (**Figure 3B**). We found that increased expression (1.7- and 2.7-fold increase, respectively) of Alix and CD63 mRNA was detected in DDX3-knockdown HCC cells compared to that of control cells. However, there was no obvious change in the mRNA level of other exosomal markers. Therefore, DDX3 may transcriptionally up-regulate the expression of Alix and CD63.

In addition, previous studies have shown that Rab proteins are widely involved in vesicle formation and protein transport through their GTPase activity [36-38]. In the process of exosome biogenesis, Rab proteins are closely related to early endosome formation and maturation, cargo sorting, vesicle tethering and fusion, vesicular transport, endosome recycling, and signaling molecule recruitment [11, 12, 21, 22]. To investigate whether Rab proteins participate in DDX3 knockdown-induced enhancement of exosome secretion in HCC cells, we examined the cellular level of Rab proteins that are relevant to the regulation of exosome biogenesis and release mechanisms, such as Rab5, Rab7, Rab11, Rab27, Rab35, and their isoforms. Increased expression of Rab5a, Rab5b, Rab11a, Rab11b, and Rab35 was

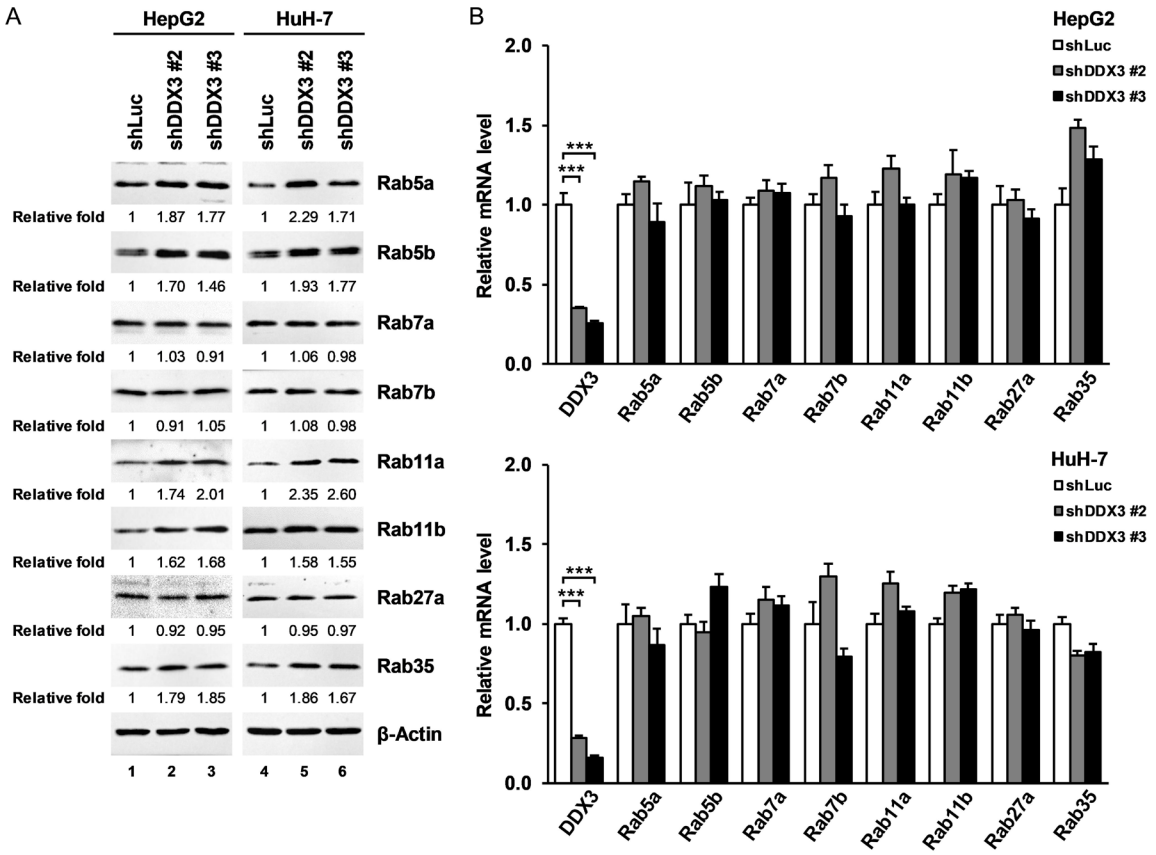

Figure 4. Decreased cellular levels of DDX3 are associated with higher expression of Rab5, Rab11, and Rab35 in HCC cells. A. The cellular levels of several exosome biogenesis-related Rab proteins were analyzed in HepG2 and HuH-7 cells. Cell lysates (50 μg) of control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HCC cells were analyzed by western blotting using antibodies against Rab5a, Rab5b, Rab7a, Rab7b, Rab11a, Rab11b, Rab27a, Rab35, and β-actin. Western blot intensities were quantified and calibrated using β-actin as the loading control, followed by normalization of results to the expression level observed in control cells. B. The mRNA levels of several exosome biogenesis-related Rab proteins were analyzed in HepG2 and HuH-7 cells. The mRNA levels of DDX3, Rab5a, Rab5b, Rab7a, Rab7b, Rab11a, Rab11b, Rab27a, Rab35, and β-actin in control (shLuc) and DDX3-knockdown (shDDX3 #2 and #3) HCC cells were analyzed by qPCR. The mRNA level of each gene was calibrated with that of β-actin. Fold changes in each mRNA transcript in shDDX3 #2 and shDDX3 #3 cells were relative to that of shLuc cells. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (***, $P < 0.001$). The data shown are from one representative experiment of three independent experiments.

observed in DDX3-knockdown HepG2 or HuH-7 cells compared to control cells (**Figure 4A**). The expression of Rab7a, Rab7b, or Rab27a did not appear to be significantly altered. Therefore, up-regulation of Rab5, Rab11, and Rab35 may contribute to enhanced exosome secretion in DDX3-knockdown HCC cells.

We also used qPCR to test whether DDX3 affects the gene transcription of Rab proteins (**Figure 4B**). Our observations indicated that the mRNA transcript levels of these Rab proteins were not significantly changed in DDX3-knockdown HCC cells compared to control cells. Thus, DDX3 may affect the gene expression of Rab5, Rab11, Rab35, and their isoforms through a mechanism other than transcriptional regulation.

DDX3 participates in exosome secretion in HCC cells by regulating exosome biogenesis-related factors such as exosome markers and Rab proteins

Studies have shown that exosome markers, such as the ESCRT components TSG101 [10, 39] and Alix [40] as well as tetraspanin CD63 [41], are involved in the formation of ILV, the

endosomal form of exosomes. As the down-regulation of DDX3 promoted exosome secretion and enhanced the cellular expression of TSG101, Alix, and CD63 in HCC cells (**Figures 1D** and **3A**), DDX3 might modulate the secretion of HCC exosomes by de-regulating the expression of these exosome markers. To verify this hypothesis, the expression of TSG101, Alix, or CD63 was reduced individually in control (shTSG101, shAlix, or shCD63) or DDX3-knockdown HepG2 cells (shDDX3-shTSG101, shDDX3-shAlix, or shDDX3-shCD63). The knockdown efficiency of each cell line with different combinations of shRNA constructs was confirmed by western blotting (**Figure 5A**). The particle size and concentration of exosomes released by these cells were detected by NTA (**Figure 5B**). The mean size of the purified exosomes derived from these cells ranged from 104 to 136.4 nm. The reduced expression of TSG101, Alix, or CD63 in HepG2 cells suppressed exosome secretion (**Figure 5C**), which was consistent with previous reports and reflected their important roles in exosome production [10, 39-41]. As the expression of TSG101, Alix, and CD63 was simultaneously decreased in DDX3-knockdown HepG2 cells, the enhancement of exosome secretion induced by DDX3 knockdown was significantly reduced. These observations suggested that the DDX3 knockdown-mediated increase in exosome production was associated with the up-regulation of TSG101, Alix, and CD63.

Rab proteins are also involved in many steps of endosomal trafficking and exosome generation. In general, the function of isoform a among each type of Rab protein is relatively well studied, and Rab5a [44], Rab11a [45], and Rab35 [48] are known to regulate exosome secretion in HCC cells. As increased cellular expression of Rab5a, Rab11a, and Rab35 as well as enhanced exosome secretion was observed in DDX3-knockdown HCC cells (**Figures 1D** and **4A**), it is also interesting to know whether DDX3 affects exosome secretion by modulating the expression of these Rab proteins. To this end, a double-knockdown strategy with the combination of DDX3 down-regulation and knockdown of Rab5a, Rab11a, or Rab35 was employed. HepG2 cells with individual knockdown of Rab5a, Rab11a, or Rab35 (shRab5a, shRab11a, or shRab35) as well as DDX3-down-regulated HepG2 cells with knockdown of Rab5a, Rab11a, or Rab35 (shDDX3-shRab5a, shDDX3-shRab11a, or shDDX3-shRab35) were established. Western blotting was performed to verify the knockdown efficiency (**Figure 6A**). By using NTA analysis, our results revealed that the average size of exosomes derived from these cell lines was between 95.9 and 118.8 nm (**Figure 6B**). The secretion of exosomes was inhibited in shRab5a, shRab11a, or shRab35 HepG2 cells (**Figure 6C**), which is consistent with previous studies and the important roles of these factors in exosome biogenesis [44, 45, 48]. Notably, DDX3 knockdown-induced enhancement of exosome secretion was significantly reduced in shDDX3-shRab5a, shDDX3-shRab11a, or shDDX3-shRab35 cells, indicating that the up-regulation of Rab5a, Rab11a, and Rab35 in HCC cells may contribute to the enhancement of exosome secretion in a DDX3-knockdown background.

The inhibitory effects of DDX3 on cancer stem cell properties of HCC cells can be transmitted to recipient cells through exosomes

Our previous study revealed that DDX3 inhibits stem cell properties, such as self-renewal ability, migration, and drug resistance, and acts as a tumor suppressor in HCC cells [87]. To examine whether the effects of DDX3 on cancer stemness properties can be transmitted between HCC cells, we treated parental HepG2 or HuH-7 cells with exosomes derived from control (shLuc-exo) or DDX3-knockdown HepG2 cells (shDDX3 #2-exo and shDDX3 #3-exo) and evaluated the cancer stemness capabilities, such as the self-renewal, migration, and drug resistance abilities of these exosome-treated HCC cells. For the assessment of self-renewal ability (**Figure 7A**), our results demonstrated that approximately 2.5-fold enrichment of formed spheres was observed in shDDX3 #2-exo and shDDX3 #3-exo HepG2 cells compared to that of shLuc-exo HepG2 cells. Similar results in parental HuH-7 cells treated with exosomes were found, in which approximately 3.3- to 3.8-fold enhancement of sphere formation was observed in shDDX3 #2-exo and shDDX3 #3-exo HuH-7 cells compared with shLuc-exo HuH-7 cells (**Figure 7B**). In the migration assay, 2.4- to 2.7-fold enhancement of migration capability in shDDX3 #2-exo and shDDX3 #3-exo HepG2 cells was observed compared with the shLuc-exo group (**Figure 7C**). The

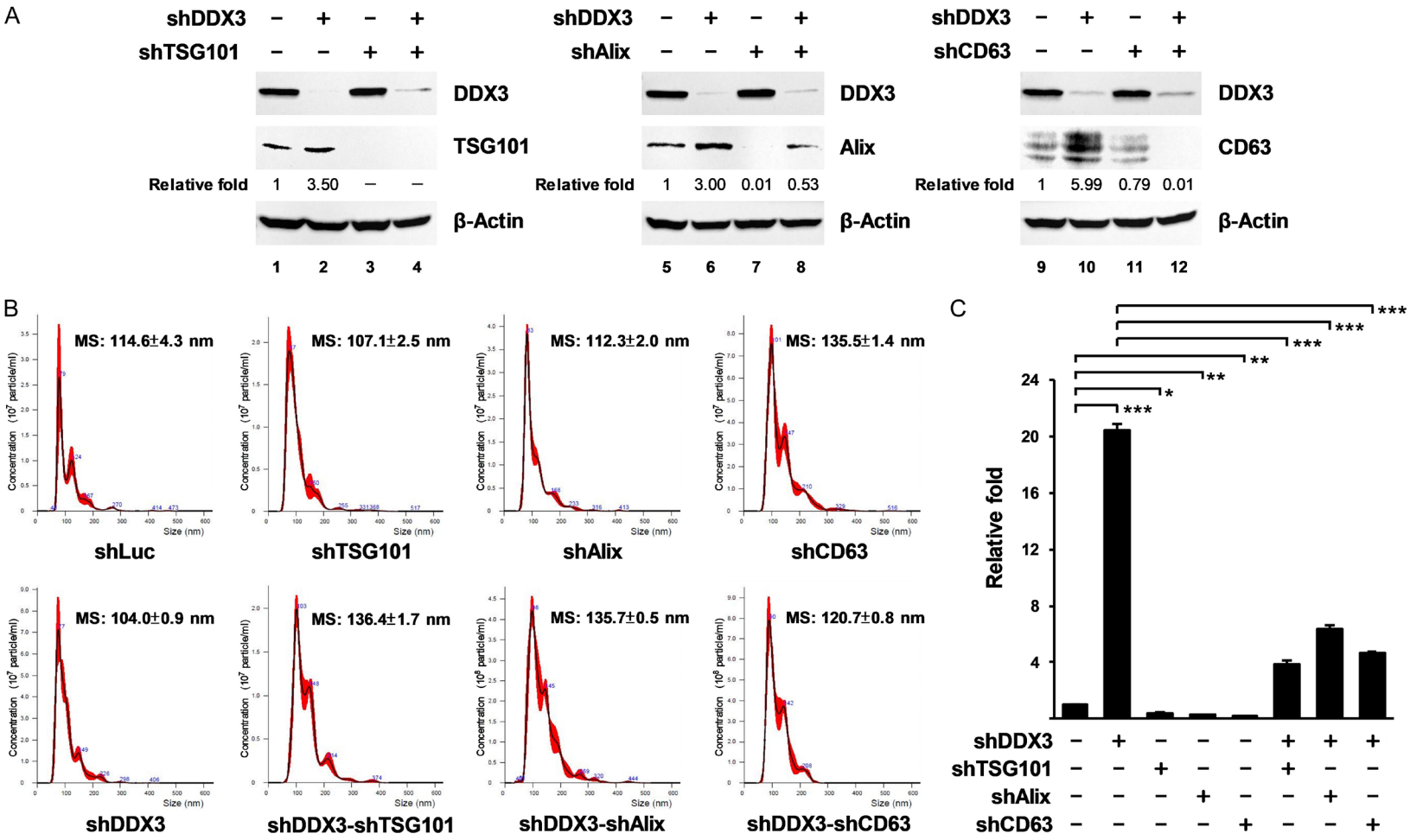

Figure 5. DDX3 participates in exosome secretion by regulating the expression of TSG101, Alix, and CD63. A. The establishment of control and single or double knockdown of DDX3 (shDDX3) or exosome markers (shTSG101, shAlix, and shCD63) in HepG2 cells. Cell lysates (50 μg) were analyzed by western blotting using anti-DDX3, TSG101, Alix, CD63 and β-actin antibodies. The western blot intensities were quantified and calibrated to that of the loading control β-actin. The results were normalized to the expression level in control cells. B. The particle distribution of purified exosomes. Exosomes released from control (shLuc) and target gene-knockdown (shTSG101, shAlix, shCD63, shDDX3 #3, shDDX3 #3-shTSG101, shDDX3 #3-shAlix, and shDDX3 #3-shCD63) HepG2 cells were purified with ultracentrifugation, and then NTA was performed to examine the size distribution of exosomes. C. DDX3 is involved in exosome secretion through the modulation of TSG101, Alix, and CD63 expression in HepG2 cells. The concentration of exosomes was analyzed by NTA, and then the number of exosome particles/cell was calculated as described in the legend of **Figure 1D**. The number of exosomes secreted per cell was transformed into the fold change relative to that of control cells. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The presented data are from a single experiment that is representative of three independent experiments. MS, mean size.

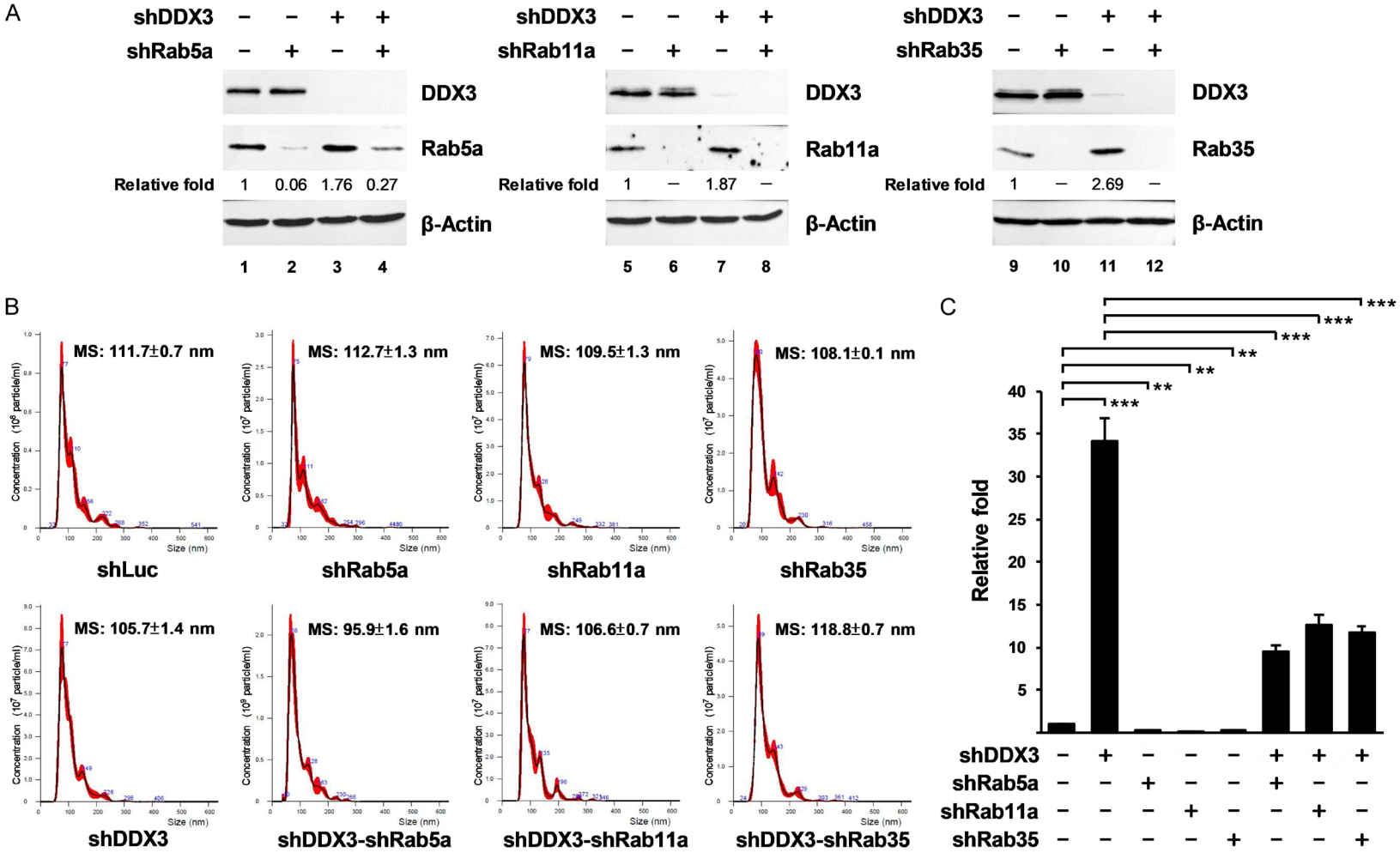

Figure 6. DDX3 regulates exosome secretion by modulating the expression of Rab5a, Rab11a, and Rab35. A. Establishment of control and single or double knockdown of DDX3 (shDDX3) or Rab proteins (shRab5a, shRab11a, and shRab35) in HepG2 cells. Cell lysates (50 μg) were analyzed by western blotting using anti-DDX3, Rab5a, Rab11a, Rab35 and β-actin antibodies. Western blot intensities were quantified and calibrated using β-actin as the loading control, followed by normalization of results to the expression level observed in control cells. B. The particle size profiles of secreted exosomes. Ultracentrifugation was performed to isolate exosomes released from control (shLuc) and target gene-knockdown (shRab5a, shRab11a, shRab35, shDDX3 #3, shDDX3 #3-shRab5a, shDDX3 #3-shRab11a, and shDDX3 #3-shRab35) HepG2 cells, and then NTA was used to detect the size distribution of exosomes. C. The secretion of exosomes involved the regulation of Rab5a, Rab11a, and Rab35 expression by DDX3. Exosome concentrations were analyzed with NTA. The number of exosome particles/cell was calculated as described in the legend of **Figure 1D**. The number of exosomes secreted per cell was transformed into the fold change relative to that of control cells. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (**, $P < 0.01$; ***, $P < 0.001$). One of three independent experiments is shown as a representative example of the data. MS, mean size.

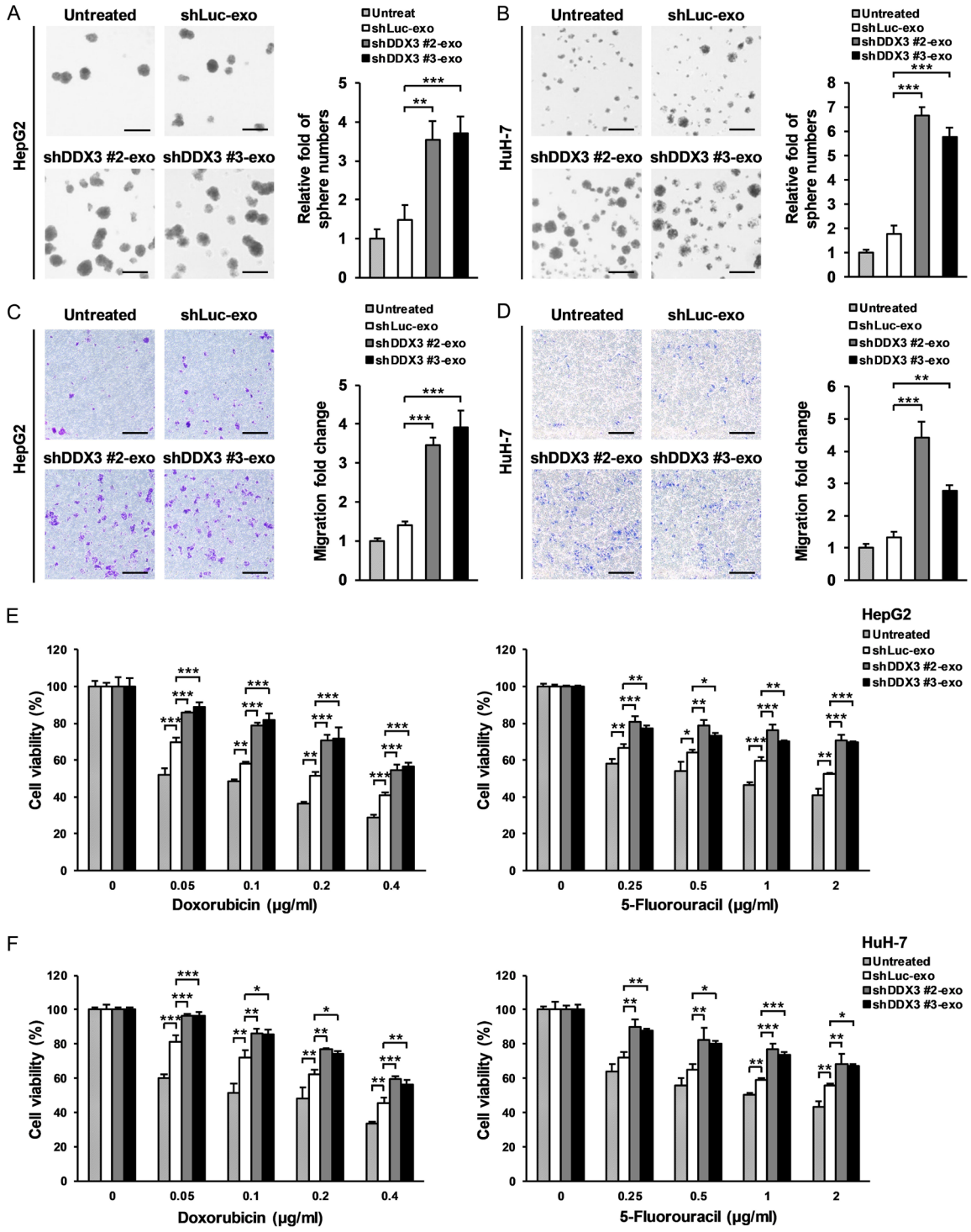

Figure 7. Exosomes derived from DDX3-knockdown cells promote the self-renewal, migration, and drug resistance of recipient HCC cells. Parental HepG2 or HuH-7 cells were treated with exosomes secreted from control or DDX3-knockdown HepG2 cells (shLuc-exo, shDDX3 #2-exo, and shDDX3 #3-exo). Parental HepG2 or HuH-7 cells treated with serum-free medium were used as mock controls (Untreated). A, B. The number of formed spheres was significantly increased in recipient HCC cells treated with exosomes from DDX3-knockdown HepG2 cells. For the sphere formation assay, exosome-treated HepG2 (5×10^3) or HuH-7 (2×10^4) cells were plated in Ultra-Low Attachment 6-well plates for 7 to 10 days. C, D. Exosomes from DDX3-knockdown HepG2 cells stimulated the migration ability of recipient HCC cells. The migration ability of exosome-treated HepG2 (1×10^5 cells/insert) or HuH-7 (2×10^4 cells/insert) cells was analyzed by migration assay. The numbers of formed spheres or migrated cells in shLuc-exo, shDDX3 #2-

exo, and shDDX3 #3-exo were calculated and transformed into fold change relative to that of untreated cells. Scale bar is equal to 200 μm. Images of formed spheres or stained migrated cells were captured as described in the Materials and Methods section. E, F. Exosomes secreted by DDX3-knockdown HepG2 cells increased the drug resistance of recipient HCC cells. Untreated, shLuc-exo-, shDDX3 #2-exo-, and shDDX3 #3-exo-treated HepG2 (1×10^4 cells/well in a 96-well plate) or HuH-7 cells (5×10^3 cells/well) were incubated with different concentrations of doxorubicin (0, 0.05, 0.1, 0.2, 0.4 μg/ml) or 5-fluorouracil (0, 0.25, 0.5, 1, 2 μg/ml), and cell viability was determined by MTS assay. The formazan absorbance at 490 nm of cells not treated with anti-cancer drugs was arbitrarily set as 100% viability. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The presented data are from a single experiment that is representative of three independent experiments.

enhancement (2.1- to 3.3-fold increase) of migrated cells was also detected in shDDX3 #2-exo and shDDX3 #3-exo HuH-7 cells compared to that of the shLuc-exo group (**Figure 7D**). For chemoresistance, HepG2 or HuH-7 cells treated with DDX3-knockdown cell-derived exosomes were incubated with different concentrations of two anti-cancer drugs, doxorubicin and 5-fluorouracil, and then the viability of the exosome-treated cells was evaluated with the MTS assay. The results indicated that shDDX3 #2-exo and shDDX3 #3-exo HCC cells were more viable than shLuc-exo cells at different concentrations of doxorubicin or 5-fluorouracil (**Figure 7E** and **7F**). Collectively, our observations revealed that the tumor-suppressive function of DDX3 in HCC could be delivered to recipient cells, possibly through exosomes.

DDX3 may exert its tumor-suppressive effect in HCC by affecting the miRNA content in exosomes

Our previous study demonstrated that DDX3 prevents the generation of hepatic cancer stem cells by epigenetically regulating a subset of tumor-suppressive microRNAs, including miR-200b, miR-200c, miR-122, and miR-145 [87]. To investigate whether the transmission of the DDX3-mediated inhibitory effect on HCC cancer stemness to recipient cells was associated with the alteration of exosome composition, the levels of miR-200b, miR-200c, miR-122, and miR-145 in exosomes derived from control and DDX3-knockdown HepG2 cells were analyzed by using qPCR. Consistent with our previous report, the cellular expression of miR-200b, miR-200c, miR-122, and miR-145 in DDX3-knockdown HepG2 cells was significantly decreased compared to that of shLuc HepG2 cells (**Figure 8A**). However, the expression of miR-200b and miR-200c in exosomes derived from DDX3-knockdown HepG2 cells was significantly reduced, while miR-122 and miR-145 were not significantly altered (**Figure 8B**). These observations suggested that DDX3 de-regulates the levels of miR-200b and miR-200c in exosomes released from HCC cells, which may contribute to the transmission of its tumor-suppressive property to recipient HCC cells.

Discussion

In this study, our results supported that DDX3 functions as a negative regulator in HCC exosome biogenesis. We found that DDX3 knockdown promoted exosome secretion by HCC cells (**Figure 1**), and the overexpression of DDX3 showed the opposite effect (**Figure 2**). The cellular levels of several exosome markers (TSG101, Alix, CD63) and Rab proteins (Rab5, Rab11, Rab35, and their isoforms) were increased in DDX3-knockdown HCC cells (**Figures 3** and **4**), which contributed to the enhancement of exosome production induced by DDX3 down-regulation (**Figures 5** and **6**). As the mRNA transcripts of Alix and CD63 were up-regulated in DDX3-knockdown HCC cells, DDX3 may regulate the expression of Alix and CD63 at the transcriptional level (**Figure 3B**). Exosomes derived from DDX3-knockdown HCC cells enhanced the hepatic cancer stemness properties of recipient cells, including self-renewal ability, migration, and drug resistance (**Figure 7**), suggesting that the tumor-suppressive function of DDX3 in HCC can be transmitted to recipient cells through exosomes. In addition, enhanced levels of TSG101, Alix, and CD63 (**Figure 3A**) as well as reduced levels of tumor-suppressive miR-200b and miR-200c (**Figure 8**) were observed in exosomes secreted by DDX3-knockdown HCC cells, implying that DDX3 possesses the ability to regulate the composition of HCC exosomes. Taken together, DDX3 knockdown stimulated exosome secretion of HCC cells, de-regulated the cargo that was sorted into HCC exosomes, and promoted the development of the hepatic stemness phe-

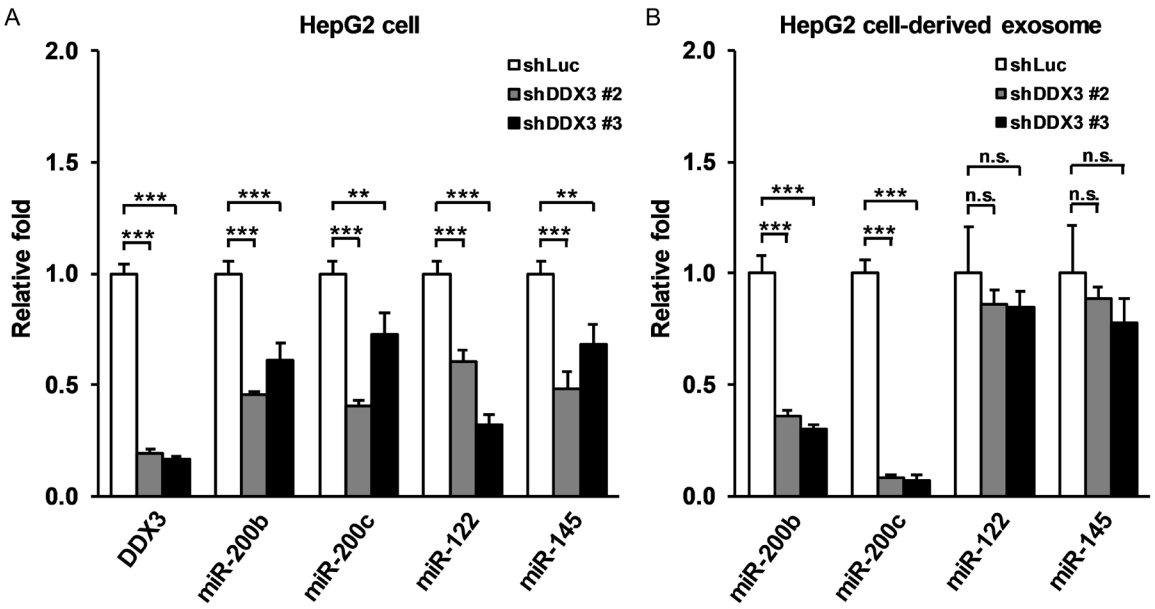

Figure 8. Lower DDX3 expression affects the levels of exosomal tumor-suppressive miRNAs. A. The levels of miR-200b, miR-200c, miR-122, and miR-145 were decreased in DDX3-knockdown HepG2 cells. The expression of tumor-suppressive miRNA (miR-200b, miR-200c, miR-122 and miR-145) and U6 snRNA in control (shLuc) and DDX3-knockdown (shDDX3 #2 and shDDX3 #3) HepG2 cells was analyzed by qPCR. B. Decreased expression of miR-200b and miR-200c was observed in exosomes secreted from DDX3-knockdown HepG2 cells. qPCR was performed to detect the expression of tumor-suppressive miRNA and U6 snRNA in exosomes secreted from DDX3-knockdown HepG2 cells. Each miRNA expression level was normalized to U6 snRNA. Fold change of each transcript in shDDX3 #2 and shDDX3 #3 group were relative to that of shLuc groups. The error bar indicates ± 1 s.d. of the mean. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc test with SigmaPlot software (n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$). One of three independent experiments is shown as a representative example of the data.

notype (**Figure 9**), which may partially account for and further strengthen its tumor-suppressive role in the progression of HCC.

Evidence from extensive studies has shown that exosomes play a key role in the communication between cancer cells and the tumor microenvironment (TME), thereby promoting tumor development and progression [8, 21, 22, 31]. For example, a previous study reported that gemcitabine treatment promotes exosome secretion from cancer-associated fibroblasts (CAFs), which leads to chemoresistance and cell proliferation of adjacent recipient pancreatic ductal adenocarcinoma (PDAC) cells. Furthermore, blocking the release of exosomes from CAFs by the exosome inhibitor GW4869 increases the gemcitabine susceptibility of recipient PDAC cells and inhibits the survival, proliferation, and tumor growth rate of recipient PDAC cells [90]. In liver cancer stem cells, the reduction in Rab27a expression suppresses the release of exosomes and inhibits Nanog expression and regorafenib resistance in recipient liver cancer cells [46]. Thus, exosomes play important roles in stimulating tumor progression and maintaining cancer stemness, and blocking the secretion of exosomes may inhibit the development of certain cancer types, including HCC. In this study, our results indicated that DDX3 inhibited exosome secretion in HCC cells (**Figures 1** and **2**), which may contribute to the de-regulation of hepatic cancer stemness properties, including self-renewal, migration, and drug resistance, in recipient HCC cells (**Figure 7**). Therefore, DDX3 may prevent the alteration of the TME caused by cancer cell-derived exosomes and inhibit HCC development, which may contribute to the tumor-suppressive role of DDX3 in HCC [58, 87, 88].

Previous studies have demonstrated that regulators of exosome biogenesis are involved in cancer progression. For example, TSG101 is overexpressed in HCC, and its lower expression suppresses the proliferation, migration, and invasion abilities of HCC cells [91, 92]. Moreover, the expression of Alix is increased and

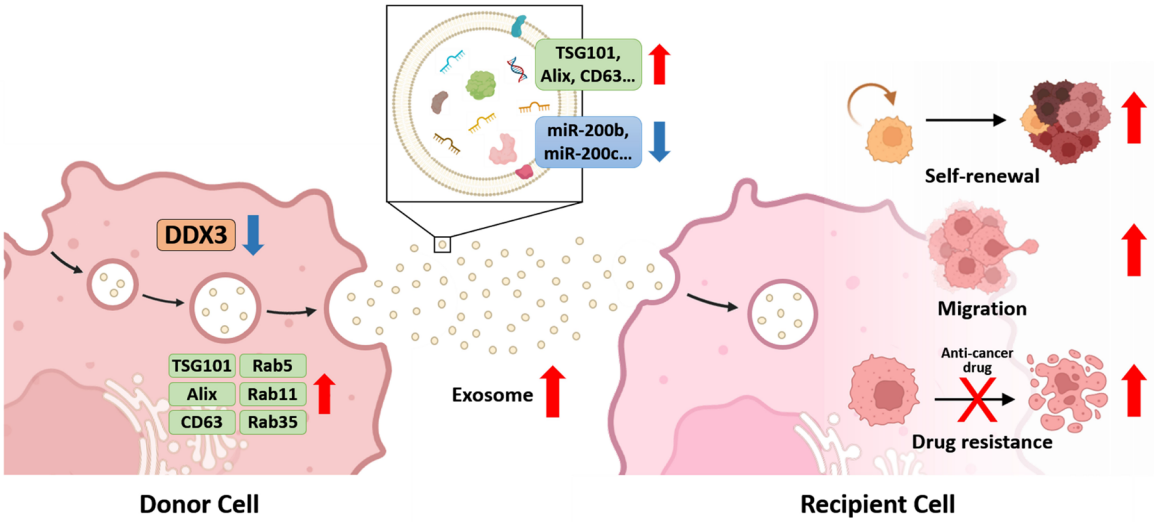

Figure 9. A proposed model showing that DDX3 down-regulation enhances HCC progression by modulating the biogenesis and composition of exosomes. DDX3 knockdown enhances the expression of several exosome biogenesis-related factors, such as exosome markers (TSG101, Alix, and CD63) and Rab proteins (Rab5, Rab11, and Rab35), thereby increasing exosome secretion by HCC cells. Increased levels of TSG101, Alix, and CD63 as well as reduced levels of tumor-suppressive miR-200b and miR-200c were found in exosomes derived from DDX3-knockdown HCC cells, which may account for the enhanced hepatic cancer stemness properties of the recipient cells. Therefore, DDX3 possesses the ability to regulate the secretion and composition of HCC exosomes, and its tumor-suppressive function in HCC progression can be transmitted to recipient cells through exosomes.

closely related to distant metastasis and TNM stage in patients with pancreatic cancer [93], but in breast cancer, Alix deficiency promotes tumor growth and makes the environment more immunosuppressive [94]. Several exosomal tetraspanins have also been reported to enhance cell motility, cell growth, angiogenesis, and metastasis in multiple cancers [95]. CD63, a type of tetraspanin that represents one of the classic exosome markers, was used as a predicted signature of an EMT-associated secretory phenotype, and its overexpression is associated with poor prognosis in patients with colorectal cancer [96]. In addition, Rab proteins, such as Rab5a [49, 50] and Rab11a [51-53], are overexpressed in HCC patients and in HCC cell lines, which promotes the proliferation and migration of HCC cells. Rab35 has been reported to be involved in immune regulation and as an upstream regulator of the PI3K/Akt pathway, which promotes the progression of leukemia [97]. However, another study showed that the inhibition of Rab35 by miR-720 promotes the migration of HeLa cells [98]. These results indicate that exosome biogenesis-related proteins may have distinct functions, either oncogenic or tumor-suppressive functions, in different types of cancers. In addition, it has been shown that exosome biogenesis-related factors, such as the exosome markers TSG101, Alix, and CD63 as well as the Rab proteins Rab5a, Rab11a, and Rab35 are enriched in exosomes [99, 100]. Our results revealed that DDX3 knockdown up-regulated the expression of TSG101, Alix, CD63, Rab5a, Rab11a, and Rab35 (**Figures 3** and **4**), which resulted in enhanced hepatic exosome secretion (**Figure 1**) and promoted cancer stemness in recipient HCC cells (**Figure 7**). These findings were consistent with the regulatory functions of these exosome biogenesis-related factors in cancer development.

DDX3 possesses the ability to regulate gene transcription. For example, DDX3 increases the activity of the $p21^{waf1/cip1}$ promoter by interacting with the transcription factor Sp1 [58]. DDX3 also binds to the E-cadherin promoter directly or as one of the components of the heterogeneous complex, which represses E-cadherin expression [59]. Moreover, DDX3 cooperates with TBK1 to synergistically activate the IFN-β promoter [62]. In addition to transcriptional regulation, DDX3 has been reported to be involved in the regulation of protein translation [68]. For example, DDX3 promotes the transla-

tion of GC-rich structures or translation-inefficient mRNAs by binding to the small subunit of the ribosomes [67]. DDX3 also stimulates the translation of mRNA containing long or structured 5'-untranslated regions (5'UTR), such as TGF-β1 [63]. Notably, a previous study indicated that lncRNA PRR34-AS1 recruits DDX3X to regulate Rab27a mRNA stability in HCC [101]. In this study, we found that lower expression of DDX3 promoted the mRNA expression of Alix and CD63, which indicated that the expression of Alix and CD63 may be regulated by DDX3 at the transcriptional level (**Figure 3B**). However, whether DDX3 directly binds to the promoters of Alix and CD63 or needs to cooperate with other transcription regulatory factors is an important issue to be addressed. As the mRNA expression of TSG101, Rab5, Rab11, or Rab35 was not significantly affected by DDX3 (**Figures 3B** and **4B**), DDX3 may regulate the expression of these exosome biogenesis-related factors through mechanisms other than transcriptional regulation.

The effects of exosomes on recipient cells depend on their composition. Several instances indicate that oncogenes or tumor suppressors actively participate in regulating the composition of exosomes secreted from cancer cells. For example, the oncogene KRAS selectively alters miRNA in exosomes secreted from colorectal cancer cells. miR-10b was increased in wild-type KRAS-derived exosomes, while miR-100 was enriched in mutant KRAS-derived exosomes [102]. In prostate cancer, non-caveolar caveolin-1 drives hnRNPK localization to MVBs and recruits cancer metastasis-promoting miRNAs containing the AsUGnA motif into exosomes [103]. In our study, exosomes derived-from DDX3-knockdown HepG2 cells enhanced the stem cell properties of recipient cells (**Figure 7**), and exosome markers TSG101, Alix, and CD63 were up-regulated while tumor-suppressive miR-200b and miR-200c were down-related in exosomes released from DDX3-knockdown HCC cells (**Figures 3A** and **8**). These findings indicated that DDX3 may regulate the levels of certain miRNAs and cellular factors in exosomes secreted from HCC cells, thereby affecting the biological functions of recipient cells. Thus, DDX3 may participate in cargo sorting of exosomes, and the composition of exosomes secreted from HCC cells with different levels of DDX3 may be distinctive.

Exosomes transmit substances between cells and the microenvironment and regulate various physiological and pathological processes, which have been widely investigated in various aspects of biological research, including cancer biology. Previous studies have indicated that exosomes are involved in the occurrence and progression of various liver diseases and liver cancer [31-33]. Due to the lack of clear biomarkers in the early stage of HCC diagnosis, the analysis of HCC exosome contents is considered to be a suitable diagnostic tool [32, 33]. In this study, we found that DDX3 inhibits HCC progression by suppressing the release of exosomes and modulating the composition of HCC exosomes. These observations may provide insight for the development of new therapeutic strategies and early diagnostic biomarkers for HCC based on the expression level of DDX3.

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Disclosure of conflict of interest

None.

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Table S1. Sequences of shRNA expression constructs targeting the coding sequences of specific genes

Construct	Target sequence
shLuc	5'-CTTCGAAATGTCCGTTCGGTT-3
shDDX3 #2	5'-CGGAGTGATTACGATGGCATT-3'
shDDX3 #3	5'-CGTAGAATAGTCGAACAAGAT-3'
shTSG101	5'-GCAGAGCTCAATGCCTTGAAA-3'
shAlix	5'-GCTGCTAAACATTACCAGTTT-3'
shCD63	5'-GTCCTGAGTCAGACCATAATC-3'
shRab5a	5'-CAAGGCCGACCTAGCAAATAA-3'
shRab11a	5'-AGTTGTCCTTATTGGAGATTC-3'
shRab35	5'-TGATGATGTGTGCCGAATATT-3'

Table S2. qPCR primers for mRNA expression

Gene	Primer sequence
DDX3	F: GAAGCTACTAAAGGTTTCTAC R: TCTCAACATCACTGAAACTTTC
TSG101	F: TTCTCAGCCTCCTGTGACCACT R: CCATTTCCTCCTTCATCCGCCA
Alix	F: GCTCAGATGAGAGAAGCCACCA R: AGTCTGGATGCCTCCCTGTTCA
CD63	F: CAACCACACTGCTTCGATCCTG R: GACTCGGTTCTTCGACATGGAAG
Hsc70	F: TCCTACCAAGCAGACACAGACC R: CAGGAGGTATGCCTGTGAGTTC
Hsp90	F: TCTGCCTCTGGTGATGAGATGG R: CGTTCCACAAAGGCTGAGTTAGC
Rab5a	F: ACTTCTGGGAGAGTCCGCTGTT R: GTGTCATCAAGACATACAGTTTGG
Rab5b	F: GGAGACTTCAGCCAAGACAGCT R: ACACTGGCTCTTGTTCTGCTGG
Rab7a	F: GTGATGGTGGATGACAGGCTAG R: AGTCTGCACCTCTGTAGAAGGC
Rab7b	F: ATATCTGGCGGGGTGATGTCCT R: CTCTCTACACCAGCCTTGAGCT
Rab11a	F: AGCACCATTGGAGTAGAGTTTGC R: AAGGCACCTACAGCTCCACGAT
Rab11b	F: TCATCGAGACCTCAGCCTTGGA R: CTGCGATCTGTTTCTGTGACACG
Rab27a	F: GAAGCCATAGCACTCGCAGAGA R: CAGGACTTGTCCACACACCGTT
Rab35	F: CAGCCCATCTTACTGCAAGCAG R: GCTGACAACCTGTCGGAGAGAA
β-Actin	F: CACCATTGGCAATGAGCGGTTC R: AGGTCTTTGCGGATGTCCACGT