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DEAD box protein DDX1 promotes colorectal tumorigenesis through transcriptional activation of the *LGR5* gene

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DDX1, a member of the DEAD box RNA helicase family, plays a critical role in testicular tumors. However, it remains to be clarified whether DDX1 is involved in other types of malignant tumors such as colorectal cancer. We disrupted the *DDX1* gene in a human colorectal cancer cell line LoVo using the CRISPR/Cas9-mediated gene-targeting system. DDX1-KO LoVo cells exhibited a much slower growth rate, produced fewer colonies in soft agar medium, and generated smaller solid tumors in nude mice than parental LoVo cells. Such phenotypes of the DDX1-KO cells were mostly reversed by exogenous expression of DDX1. These results indicate that DDX1 is required for tumorigenicity of colorectal cancer cells. In the DDX1-KO cells, the cancer stem cell marker genes *LGR5*, *CD133*, *ALDH1* and *SOX2* were markedly suppressed. Among them, expression of LGR5, which is essential for tumorigenicity of colorectal cancer cells, was restored in the DDX1-transfected DDX1-KO cells. Consistently, the DDX1-KO cells lost sphere-forming capacity in a DDX1-dependent fashion. Reporter and chromatin immunoprecipitation assays revealed that DDX1 directly bound to the −1837 to −1662 region of the enhancer/promoter region of the human *LGR5* gene and enhanced its transcription in LoVo cells. Repression of *LGR5* by DDX1 knockdown was observed in 2 other human colorectal cancer cell lines, Colo320 and SW837. These results suggest that LGR5 is a critical effector of DDX1 in colorectal cancer cells. The DDX1-LGR5 axis could be a new drug target for this type of malignant cancer.

KEYWORDS
cancer stem cells, colorectal cancer, LGR5, RNA helicase, Tumorigenesis

1 | INTRODUCTION

Colorectal cancer is one of the leading causes of tumor-related mortality in the world. Colorectal carcinogenesis is considered to be a long-term process involving multiple genetic alterations. The adenomatous polyposis coli (*APC*) gene on chromosome 5p, *TP53* on 17p, and *SMAD4* on 18q are potential tumor suppressor genes for colorectal carcinogenesis, while *KRAS* on 12p is an oncogene.[1,2] Matano et al (2015) established an in vitro human colorectal cancer model through introduction of *APC*, *SMAD4*, *TP53* and *KRAS* mutations in the intestinal organoid culture system.[3]

Aberrant activation of the Wnt signaling pathway is a main oncogenic driver in 90% of colorectal cancer patients with *APC* mutations.[4] In normal mucosa, the β-catenin level is kept low in the cytoplasm by the action of a destruction complex composed of glycogen synthase kinase 3, Axin1, casein kinase 1, APC and other factors. Mutations in *APC* abolish the destructive function, leading to the accumulation and nuclear translocation of β-catenin and subsequent transcriptional activation of its target genes, including *LGR5*, *c-Myc* and *Cyclin-D1*.[5]

LGR5, also known as GPR49, is a member of the G protein-coupled receptor (GPCR) family. LGR5 was originally identified as a Wnt/Tcf4 target gene in colorectal cancer.[6] *LGR5* is overexpressed in colorectal,[7] ovarian,[7] hepatocellular[8] and basal cell[9] cancers. LGR5 expression was detected in human colorectal stem cells located between Paneth cells in the intestinal crypts.[10] Furthermore, cell lineage-tracing experiments demonstrated that LGR5-positive cells are intestinal cancer stem cells (CSC).[11] LGR5-positive intestinal stem cells are the cells of origin for adenoma caused by *APC* deletion[10,11] and are present inside colorectal tumors in an *Apc*-KO mouse model.[12]

DDX1 is a member of the DEAD box RNA helicase family characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD). DDX1 was originally identified by differential screening of a cDNA library enriched in transcripts present in a retinoblastoma cell line.[13] *DDX1* is coamplified with *MYCN* and overexpressed in a subset of neuroblastoma and retinoblastoma cell lines and tumors.[13,14] DDX1 is involved in a variety of biological processes, including tRNA synthesis,[15] mRNA and microRNA processing,[16] ribosome biogenesis, DNA repair,[17] and nuclear factor-kappaB-mediated gene induction.[18] Because DDX1 deficiency in mice causes early embryonic lethality, it must play essential roles in normal cells.[19]

DDX1 plays a critical role in testicular tumorigenesis in part by promoting transcription of *Cyclin-D2* and stem cell-related genes on human chromosome 12p.[20] The expression level of *DDX1* is elevated not only in germ cell tumors but also in retinoblastoma, neuroblastoma, glioblastoma and breast cancer.[21-24] However, it remains unknown whether DDX1 plays a role in colorectal carcinogenesis.

In this study, we explored the function of DDX1 in human colorectal cancers by disrupting the *DDX1* gene in a representative cell line LoVo. We showed that DDX1-KO LoVo cells have defects in colony and sphere-forming capacity in vitro and in vivo tumorigenesis in nude mice. More importantly, we demonstrated that DDX1 promotes the expression of the *LGR5* gene by direct interaction with its enhancer/promoter region. Thus, DDX1 is an important regulator of colorectal CSC.

2 | MATERIALS AND METHODS

2.1 | Cell culture

LoVo, Colo320 and SW837 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (PS; Sigma) at 37°C in humidified air with 5% CO_2.

2.2 | Gene disruption, overexpression and knockdown

Guide RNA (gRNA) sequence for the *DDX1* gene was chosen using the clustered regularly interspersed short palindromic repeat (CRISPR) Direct tool (http://crispr.dbcls.jp/). Oligodeoxynucleotide encoding *DDX1* single guide RNA (sgRNA) was inserted into the PX458 expression vector (Addgene, Cambridge, MA, USA), which bicistronically expresses sgRNA and the CRISPR-associated protein 9 (Cas9) nuclease. This was transfected into LoVo cells with Lipofectamine3000 (Thermo Fisher Scientific). After 48 hours in culture, GFP-positive cells were separated by FACS on a FACSAriaIII (BD Biosciences, San Jose, CA, USA) and cloned using steel cylinders. A DDX1-overexpressing LoVo cell clone was established using the retroviral vector pMY-IG as previously described.[25] Retroviral introduction of siRNA for *DDX1* gene was done as previously described.[20]

2.3 | Quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen, Paisley, UK). Synthesis of cDNA and quantitative PCR were carried out using PrimeScript RT Reagent Kit (Takara, Otsu, Japan), Thunderbird SYBR RT-PCR kit (Toyobo, Osaka, Japan), and a LightCycler480 System (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturers' protocols. *GAPDH* was used as an internal control. All reactions were carried out in triplicate. Sequence information of primers is shown in Table 1.

TABLE 1 Primers used for quantitative RT-PCR analysis

Gene	Forward primer	Reverse primer	Product (bp)
DDX1	5′- CAGAAGCCCTAGGTCCCAGA -3′	5′- GAGCCAATCCATCTCTTCCA -3′	287
LGR5	5′- TCCAACCTCAGCGTCTTCAC -3′	5′- CGCAAGACGTAACTCCTCCA -3′	111
CD133	5′- GAGTCGGAAACTGGCAGATAGCA -3′	5′- ACGCCTTGTCCTTGGTAGTGTTG -3′	113
ALDH1	5′- AGCCTTCACAGGATCAACAGA -3′	5′- GTCGGCATCAGCTAACACAA -3′	124
SOX2	5′- TGAGCGCCCTGCAGTACAA -3′	5′- GCTGCGAGTAGGACATGCTGTAG -3′	84
GAPDH	5′- GATCATCAGCAATGCCTCCT -3′	5′- TTCAGCTCAGGGATGACCTT -3′	240

2.4 | Western blot analysis

Protein concentration was determined using the BCA Protein Assay Reagent (Thermo Fisher Scientific), and equal amounts of protein were subjected to 10% SDS-PAGE. Standard western blot analyses were performed using anti-DDX1 (11357-1-AP, ProteinTech, Chicago, IL, USA), anti-LGR5 (ab75732, Abcam, Cambridge, UK) or anti-α-tubulin (T9026, Sigma-Aldrich, St Louis, MO, USA) antibody. Specific protein bands were detected by HRP-conjugated secondary anti-rabbit or anti-mouse antibody (#7074, #7076, Cell Signaling, Danvers, MA, USA) and visualized with Image Quant LAS 1000 (GE Healthcare, Buckinghamshire, UK).

2.5 | Xenograft experiments

Male athymic BALB/c nu/nu male mice (5 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan). LoVo cells (5×10^6) in 100 μL of 50% Matrigel (BD Biosciences) were subcutaneously injected into nude mice. Tumor size was measured with a caliper every 7 days, and the estimated volume of the tumor was calculated using the following formula: volume = $\pi/6 \times (\text{length} \times \text{width}^2)$. All mice were maintained under a 12 hours light/12 hours dark cycle in a pathogen-free animal facility. All experimental procedures involving mice were preapproved by the Ethical Committee for Animal Experiments at Tokyo Metropolitan Institute of Medical Science and were performed according to the Guidelines for the Proper Conduct of Animal Experiments.

2.6 | Sphere formation assay

LoVo cells (4×10^3) were plated onto a 35 mm ultralow attachment plate (Greiner Bio-One, Frickenhausen, Germany) in 10% FBS-DMEM, supplemented with 50 μL of HA-Matrix (Agcell, Tottori, Japan). After 6 days in culture, the number of spheres (volume > 1.2×10^4 μm^3) was counted and their volume was analyzed on a BX-X700 fluorescence microscope (Keyence, Osaka, Japan). This experiment was performed 3 times in triplicate.

2.7 | Luciferase reporter assay

A genomic DNA sequence containing the human *LGR5* gene was obtained from a public database (Ensembl Gene ID: ENSG00000139292). DNA fragments covering the enhancer/promoter region of the *LGR5* gene were PCR amplified using the genomic DNA of TIG-1 cells as a template and cloned into a pGL3 basic vector (Promega, Madison, WI, USA) using an In-Fusion HD Cloning Kit (Takara). LoVo cells (8×10^3 cells/well) in 96-well white-bottom plates (Sumitomo Bakelite, Tokyo, Japan) were transfected with each reporter construct (200 ng) in combination with the pRL-CMV vector (Promega) using Lipofectamine 3000 reagent. In some experiments, the DDX1 expression vector (pMY-IG-DDX1) or the empty vector (pMY-IG) was cotransfected. After 48 hours in culture, enzymatic activities were analyzed using the Dual-Glo Luciferase reporter assay kit (Promega) in a Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany). Results were obtained from 2 independent experiments performed in triplicate.

2.8 | Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded specimens, purchased from US Biomax (colorectal cancer tissue array, CO484a, Rockville, MD, USA). After de-waxing in xylene and graded ethanol, the section was incubated in 3% H_2O_2 solution to block endogenous peroxidase activity. The section was incubated with anti-DDX1 primary antibody (1:100) at 4°C overnight and processed using the DAB system (Vector, Burlingame, CA, USA). Paraffin sections of xenograft-derived tumors were stained with anti-PCNA antibody (1:100, M0879, Agilent, Santa Clara, CA, USA) in combination with AlexaFluor 546-conjugated anti-mouse IgG antibody (A11030, Thermo Fisher Scientific).

2.9 | Immunofluorescence analysis

Cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with anti-DDX1 or anti-LGR5 (ab75732, Abcam) antibody overnight at 4°C. They were stained with AlexaFluor 488-conjugated anti-rabbit IgG antibody (R37116, Thermo Fisher Scientific) and analyzed on a TSC SP8 Confocal microscope (Leica Microsystems, Mannheim, Germany).

2.10 | ChIP assay

A cross-linked chromatin fraction of LoVo cells was subjected to immunoprecipitation using anti-DDX1 or anti-rabbit IgG antibody (ab172730, Abcam) as previously described.[20] The amount of target genomic DNA in the immunoprecipitate was examined by real-time PCR on a LightCycler480. Sequence information of primers is shown in Table 2.

2.11 | Statistical analysis

All statistical analyses were performed using Microsoft Excel. Differences between individual groups were analyzed using a 2-tailed unpaired *t* test. A *P*-value of <.05 was considered significant.

TABLE 2 Primers used for ChIP analysis

Site	Forward primer	Reverse primer	Product (bp)
LGR5-P1	5′- CAGAGTGTTAAAACGGATTTT -3	5′- AGTGCACTCATCACCCAAGT -3′	112
LGR5-P2	5′- GGCCCAGCTTGAATTACTCC -3′	5′- TTCAACATGTGACCTGAGTGC -3′	179

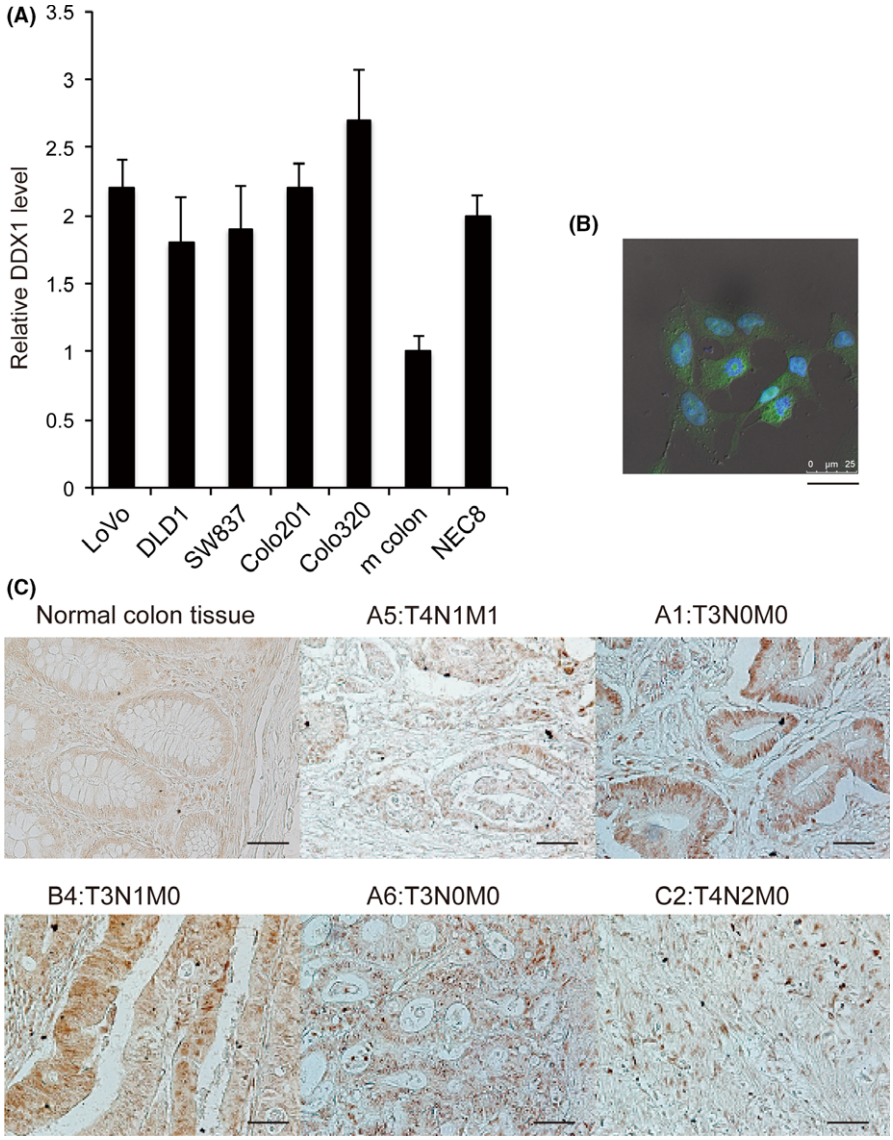


FIGURE 1 Expression of DDX1 in colorectal cancer cell lines and specimens. A, Expression levels of *DDX1* mRNA in various human colorectal cancer cell lines were determined by quantitative RT-PCR. Mouse (m) colon and a human testicular germ cell line NEC8 were used as normal tissue and cancer controls, respectively. *GAPDH* levels were used for normalization. Each value represents the mean (n = 3) ± standard deviation (SD). B, Immunofluorescent staining of permeabilized LoVo cells by anti-DDX1 antibody followed by AlexaFluor 488-conjugated secondary antibody. Nuclei were counterstained with DAPI. Scale bars = 25 μm. C, Immunohistochemical staining of paraffin-embedded sections of normal human colon and various stages of human colorectal adenocarcinomas. Scale bars = 50 μm. TNM grading of human colon cancers is as follows. M0, no distant metastasis; M1, distant metastasis; N0, no regional lymph node metastasis; N1, metastasis in 1-3 regional lymph nodes; N2, metastasis in 4 or more regional lymph nodes; T3, tumor invades through muscularis propria into subserosa or into nonperitonealized pericolic or perirectal tissues; T4, tumor directly invades other organs or structures and/or perforate visceral peritoneum

3 | RESULTS

3.1 | DDX1 is highly expressed in human colorectal cancer cell lines and tissues

We previously reported that DDX1 plays a critical role in testicular tumorigenesis using the human testicular germ cell tumor cell line, NEC8.[20] To examine whether DDX1 is also involved in colorectal carcinogenesis, we first compared the mRNA expression level of *DDX1* in NEC8 cells with that in 5 human colorectal cancer cell lines by quantitative RT-PCR. *DDX1* was highly expressed in all of these colorectal cancer cell lines, with expression as high as that in NEC8, and much higher than that in normal mouse colorectal tissue (Figure 1A). In LoVo cells, DDX1 protein was localized in both nucleus and cytoplasm (Figure 1B). Next, we investigated the expression of DDX1 protein in various histological subgroups of human

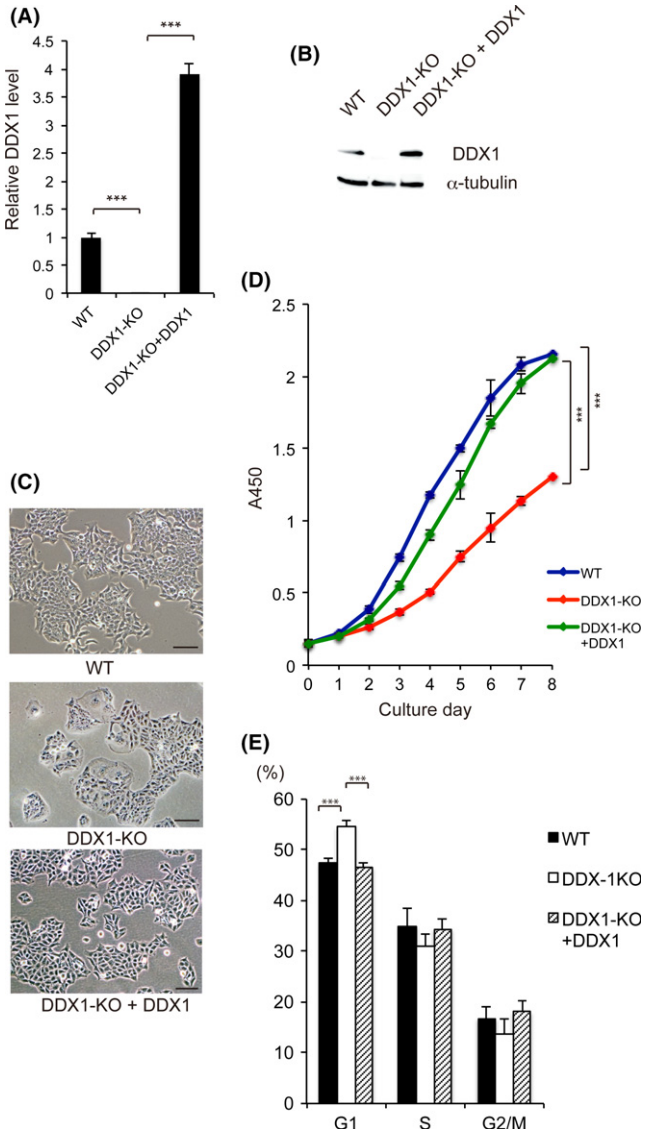


FIGURE 2 Characterization of DDX1-KO LoVo cells with or without exogenous DDX1. A, Expression levels of *DDX1* mRNA in WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells were determined by quantitative RT-PCR. *GAPDH* levels were used for normalization. Each value represents the mean (n = 3) ± SD. ***$P < .001$. B, Total cell lysates from WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells were subjected to western blot analysis with anti-DDX1 or anti-α-tubulin (control) antibody. C, Morphological appearance of WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells. Scale bars = 100 μm. D, Relative cell number of WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells in 10% FBS-DMEM was measured for 8 d using Cell Counting Kit 8. Each value represents the mean (n = 3) ± SD. ***$P < .001$. E, Cell cycle status of WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells grown in 10% FBS-DMEM was determined by FACS. Each value represents the mean (n = 4) ± SD. ***$P < .001$

colorectal cancer and normal colon. Strong signals for DDX1 were detected in these colorectal cancer patient specimens, whereas they were below the detection level in normal colorectal tissue (Figure 1C). The amount of DDX1 protein did not correlate with the pathological grade and clinical stage of the colorectal cancer.

3.2 | DDX1 is required for proliferation of the human colorectal cancer cell line LoVo

To examine whether DDX1 plays an essential role in colorectal carcinogenesis, we disrupted the *DDX1* gene of LoVo cells using CRISPR/Cas9-mediated genome editing. We cotransfected 2 gRNAs targeting the transcription start site (TSS) into LoVo cells and sorted GFP-positive cells by FACS. Through molecular characterization of *DDX1* genes in several cell clones, we established a DDX1-KO LoVo cell line carrying a 106-bp biallelic deletion (−107 to −2 relative to the TSS) in the 5′ untranscribed region of the *DDX1* gene. DDX1-KO LoVo cells did not produce DDX1 mRNA or protein (Figure 2A,B). Morphologically, DDX1-KO LoVo cells had a more flattened shape with more vacuoles than parental WT LoVo cells (Figure 2C). Importantly, the growth rate of DDX1-KO cells in regular growth medium was approximately half that of WT cells (Figure 2D).

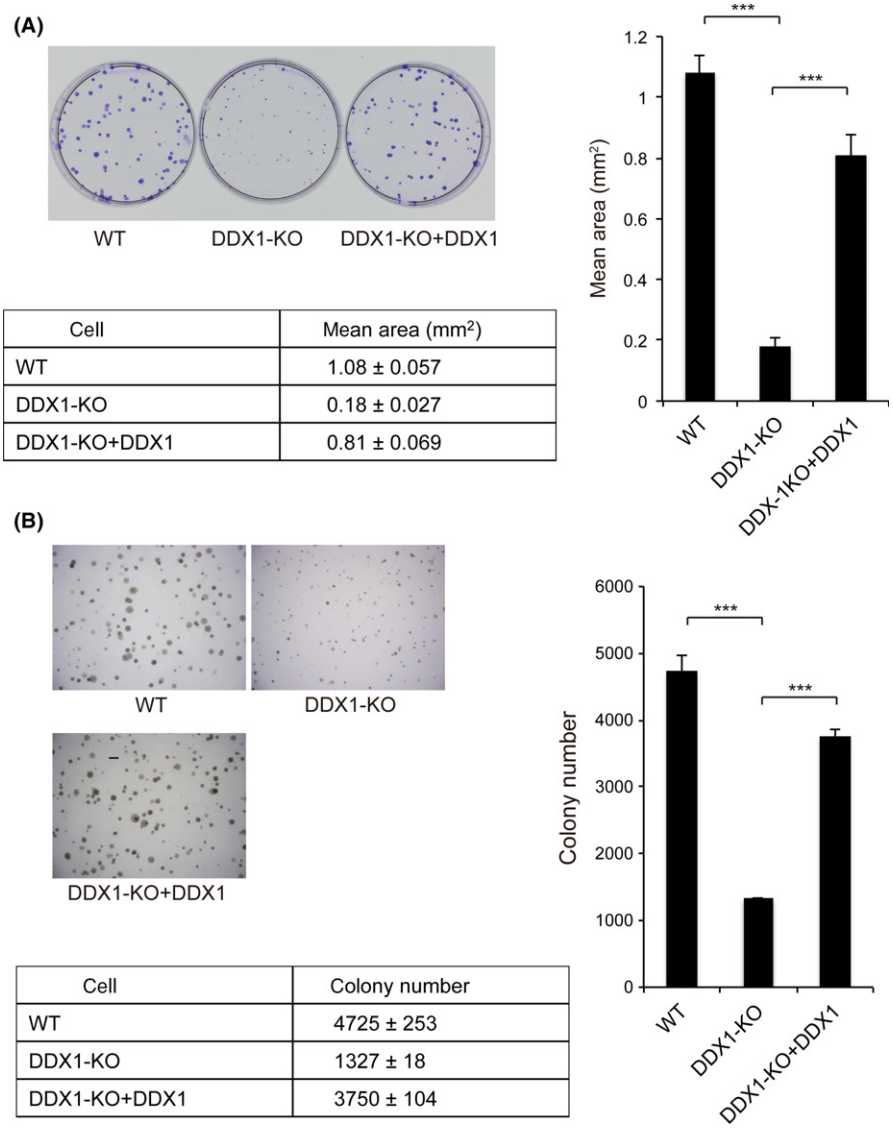
To confirm that the above phenotypic changes in DDX1-KO LoVo cells were caused by the lack of DDX1, we infected DDX1-KO cells with a DDX1 retroviral vector (pMY-IG-DDX1) to generate DDX1-KO + DDX1 LoVo cells. The mRNA expression level of *DDX1* in DDX1-KO + DDX1 cells was fourfold higher than in WT cells (Figure 2A). Accordingly, the protein level of DDX1 in DDX1-KO + DDX1 cells was higher than in WT cells (Figure 2B). DDX1-KO + DDX1 cells were morphologically indistinguishable from WT cells (Figure 2C). Most importantly, the growth rate of DDX1-KO + DDX1 cells was faster than that of DDX1-KO cells and similar to that of WT cells in 8-day culture (Figure 2D).

We next compared cell cycle status (Figure 2E). The percentage of cells in G1 phase was significantly higher in DDX1-KO cells (54.6% ± 1.1%) than in WT cells (47.4% ± 0.9%) and DDX1-KO + DDX1 cells (46.7% ± 0.7%). Reflecting this fact, the relative frequency of cells in S phase was lower in DDX1-KO cells (31.2% ± 2.3%) than in WT cells (35.1% ± 3.3%) and DDX1-KO + DDX1 cells (34.5% ± 2.0%). These data suggested that the decreased proliferation of DDX1-KO cells is due to cell cycle arrest.

3.3 | DDX1 is required for density-independent and anchorage-independent growth of LoVo cells

We next compared growth capacity of WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells under low cell density culture conditions. WT and DDX1-KO + DDX1 cells grew to form cell islands, whereas DDX1-KO cells produced fewer and much smaller cell islands (Figure 3A). The average size of DDX1-KO-derived cell islands was 16.3% that of WT cells, whereas that of DDX1-KO + DDX1-derived cell islands was 75.0% that of WT cells (Figure 3A).

Similar results were obtained in the soft agar colony formation assay. The average number of DDX1-KO-derived colonies was 28.1% that of the WT, whereas the number of DDX1-KO + DDX1-derived colonies was 79.4% that of the WT (Figure 3B). In addition, DDX1-KO-derived colonies in soft agar medium were smaller than WT-derived and DDX1-KO + DDX1-derived colonies (Figure 3B).



Cell	Mean area (mm²)
WT	1.08 ± 0.057
DDX1-KO	0.18 ± 0.027
DDX1-KO+DDX1	0.81 ± 0.069

Cell	Colony number
WT	4725 ± 253
DDX1-KO	1327 ± 18
DDX1-KO+DDX1	3750 ± 104

FIGURE 3 Colony forming capacity of DDX1-KO LoVo cells with or without exogenous DDX1. A, WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells (100 cells) were inoculated in 20% FBS-DMEM. After 10 d in culture, cells were fixed in methanol and stained with 0.1% crystal violet. The size of each colony was measured by Hybrid cell count. Each value represents the mean (n = 3) ± SD. ***$P < .001$. B, WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells (10^4 cells) were inoculated in soft agar medium. After 2 weeks in culture, the total number of colonies was counted by Hybrid cell count. Each value represents the mean (n = 3) ± SD. ***$P < .001$

These results indicated that DDX1 is required for the clonogenic proliferation of LoVo cells.

3.4 | DDX1 influences the in vivo tumorigenic capacity of LoVo cells

We next investigated a DDX1 function in tumorigenesis by employing the standard xenograft tumor mode. WT and DDX1-KO + DDX1 LoVo cells formed solid tumors of similar size under the skin of nude mice (Figure 4A-C). By contrast, the average size of DDX1-KO-derived tumors was nearly half that of those derived from WT and DDX1-KO + DDX1 (Figure 4A-C). H&E staining of the tumor sections revealed relative uniformity of the nucleus size of LoVo-derived cells (Figure 4D). Consistent with the smaller tumor size, PCNA-positive cells in the DDX1-KO + DDX1-derived tumor section were greatly decreased (Figure 4D). These results demonstrate that DDX1 plays important roles in colorectal cancer progression.

3.5 | DDX1 is required for LGR5 expression in LoVo cells

Because we previously found that DDX1 is a transcriptional activator of stem cell-related genes in human testicular tumor cells, we next examined the expression of colorectal stem cell marker genes. In DDX1-KO LoVo cells, mRNA levels of *LGR5*, *CD133*, *ALDH1* and *SOX2* genes were <10% of those in WT LoVo cells (Figure 5A). Moreover, they were mostly (*LGR5*) or partially (*CD133*, *ALDH1* and *SOX2*) restored by the exogenous DDX1

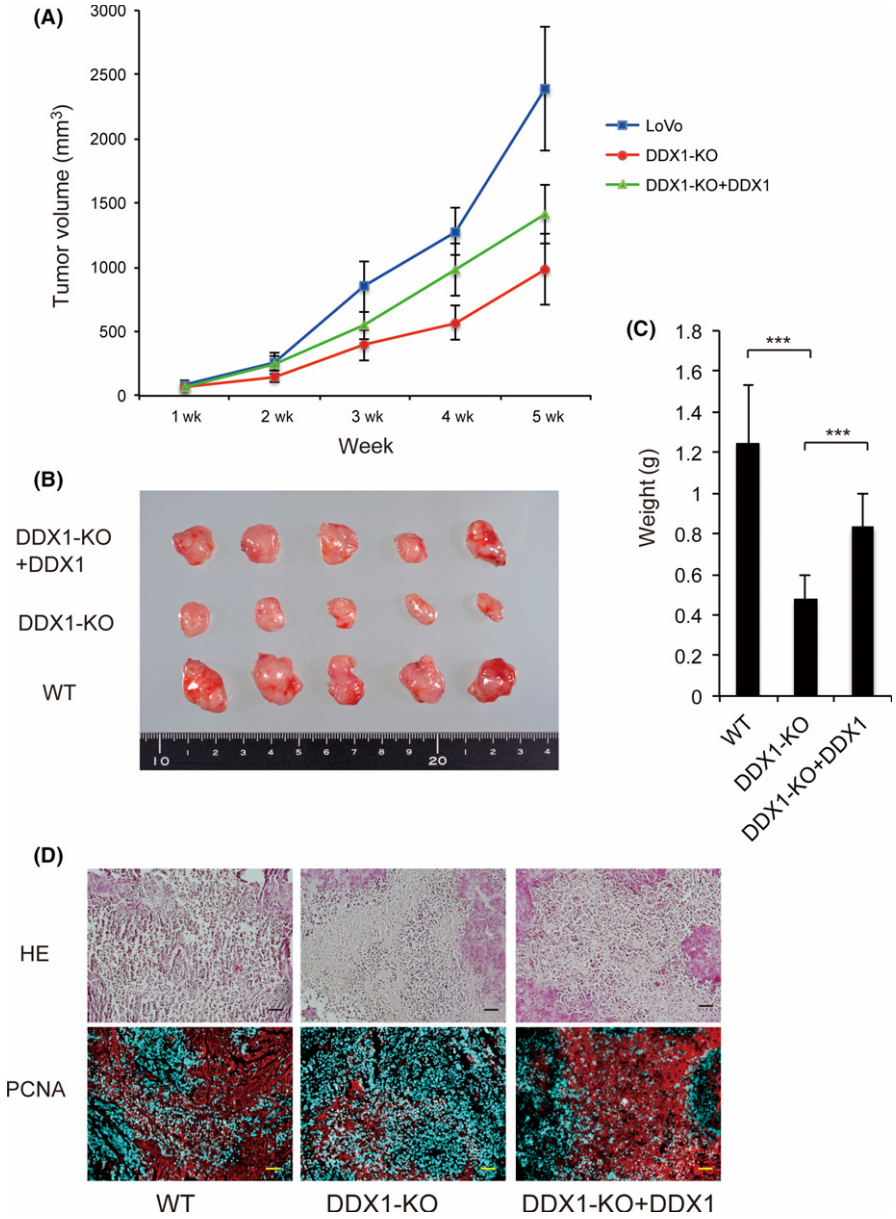


FIGURE 4 In vivo tumorigenicity of DDX1-KO LoVo cells with or without exogenous DDX1. A, WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells (5×10^6 cells) were subcutaneously injected into nude mice. The volume of each tumor was measured every week. B, Photographs of isolated tumors from the transplanted mice. C, Weight of isolated tumors from the transplanted mice. Each value represents the mean (n = 5) ± SD. ***$P < .001$. D, H&E and immunohistochemical staining data of paraffin-embedded sections of isolated tumors from the transplanted mice. Anti-PCNA antibody and DAPI were used for visualizing mitotic cells and nuclei, respectively. Scale bars = 50 μm

expression in DDX1-KO + DDX1 LoVo cells (Figure 5A). Expression of *LGR5* mRNA was repressed by siRNA-mediated knockdown of DDX1 in 2 other human colorectal cancer cell lines, Colo320 and SW837 cells (Figure 5B), excluding the possibility that this phenomenon is specific to LoVo cell line. In LoVo cells, loss of LGR5 by the DDX1 disruption and its rescue by exogenous DDX1 were detected at protein levels by western blotting (Figure 5C). Quantification of the immunofluorescent intensity of LGR5 protein in the cells also revealed such a difference between WT or DDX1-KO + DDX1 and DDX1-KO cells (Figure 5D). These results demonstrated that DDX1 is indispensable for the expression of LGR5 in LoVo cells.

LGR5-expressing cells represent colorectal CSC. To investigate the possibility that DDX1 plays a role in the maintenance of CSC, we carried out the tumor sphere formation assay. While WT LoVo cells generated approximately 240 spheres per 1000 cells, DDX1-KO LoVo cells produced eightfold fewer spheres (Figure 5E). The impaired sphere-forming capacity of DDX1-KO cells was partially restored by exogenous DDX1 expression in DDX1-KO + DDX1 cells (Figure 5E). The average volume of DDX1-KO-derived spheres also decreased to 24.3% that of WT-derived spheres. It was rescued to 45.3% by exogenous DDX1 expression in DDX1-KO + DDX1 cells (Figure 5E). These data suggest that DDX1 contributes to the sphere-forming capacity of a CSC population in LoVo cells.

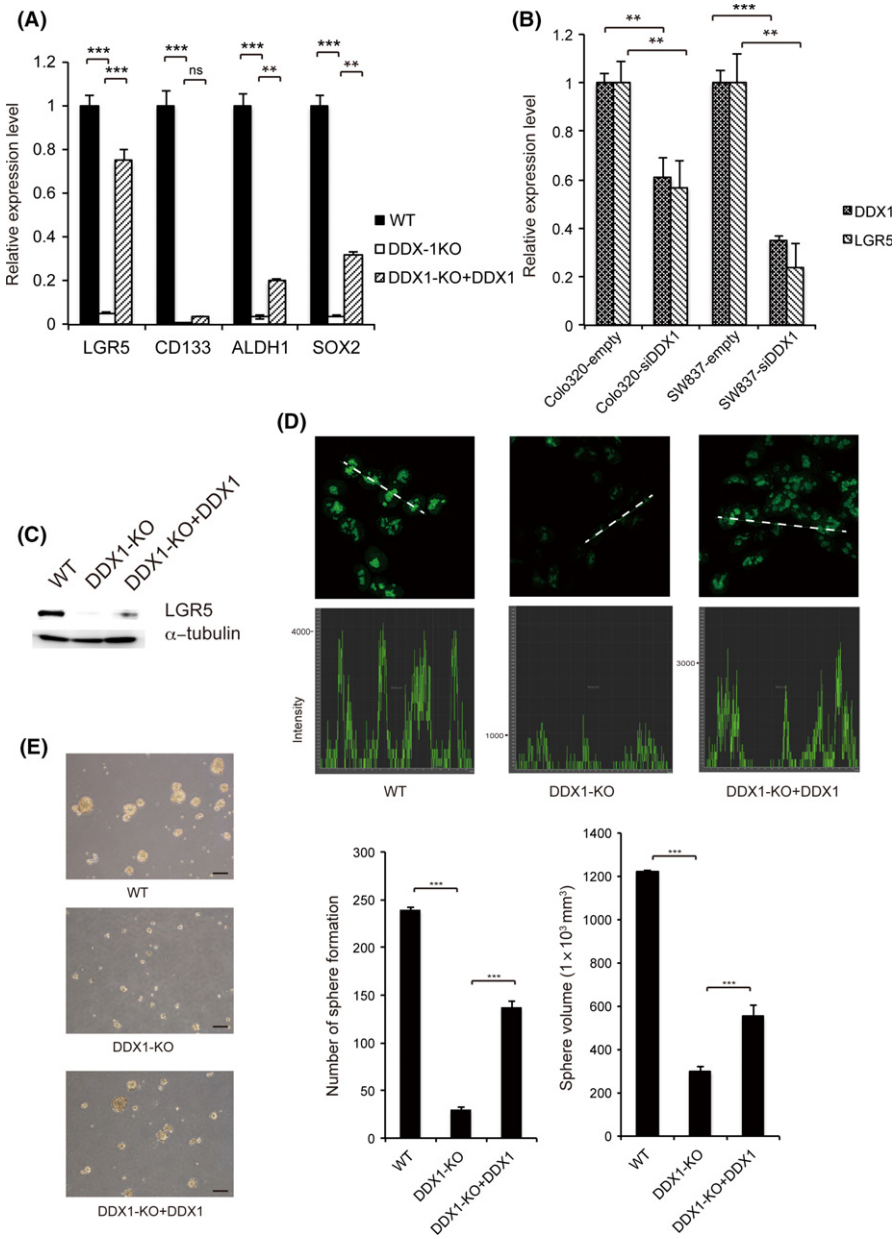


FIGURE 5 Cancer stem cell-related properties of DDX1-KO LoVo cells with or without exogenous DDX1. A, Expression levels of *LGR5*, *CD133*, *ALDH1* and *SOX2* mRNA in WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells were determined by quantitative RT-PCR. B, Colo320 and SW837 cells were transduced with *DDX1*-siRNA (siDDX1) or empty retroviral vector. Expression levels of *DDX1* and *LGR5* mRNA in these cells were determined by quantitative RT-PCR. A,B, *GAPDH* levels were used for normalization. Each value represents the mean (n = 3) ± SD. $^{**}P < .01$, $^{***}P < .001$; ns, not significant. C, Total cell lysates from WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells were subjected to western blot analysis with anti-LGR5 or anti-α-tubulin (control) antibody. D, WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells were stained with anti-LGR5 antibody followed by AlexaFluor 488-conjugated secondary antibody. Fluorescent images of the cells (upper) and histograms of the fluorescent intensity at the indicated white lines (lower) are shown. E, Morphology of spheres formed by WT, DDX1-KO and DDX1-KO + DDX1 LoVo cells after 6 d in culture. Scale bars = 200 μm. Total number of spheres per 10^3 cells (left) and their volume (right) are shown. Each value represents the mean (n = 3) ± SD. $^{***}P < .001$

3.6 | DDX1 is a transcriptional activator of the *LGR5* gene in LoVo cells

DDX1 directly binds to the enhancer/promoter region of the *cyclin-D2* gene, thereby promoting its transcription.[20] To clarify the underlying mechanism of the DDX1-dependent expression of the *LGR5* gene in LoVo cells, we generated a series of luciferase reporter constructs carrying DNA fragments of the human *LGR5* enhancer/promoter region (Figure 6A). When transfected into WT and DDX1-KO LoVo cells, LGR5-2937 and LGR5-1837 reporters displayed significantly higher values in WT cells than in DDX1-KO cells (Figure 6B). LGR5-1662, LGR5-1440 and LGR5-635 reporters showed no differences. We found that partial sequence for the DDX1-binding motif (5′-ACGTACACACCCTTTATGCC-3′) is present at 3 sites

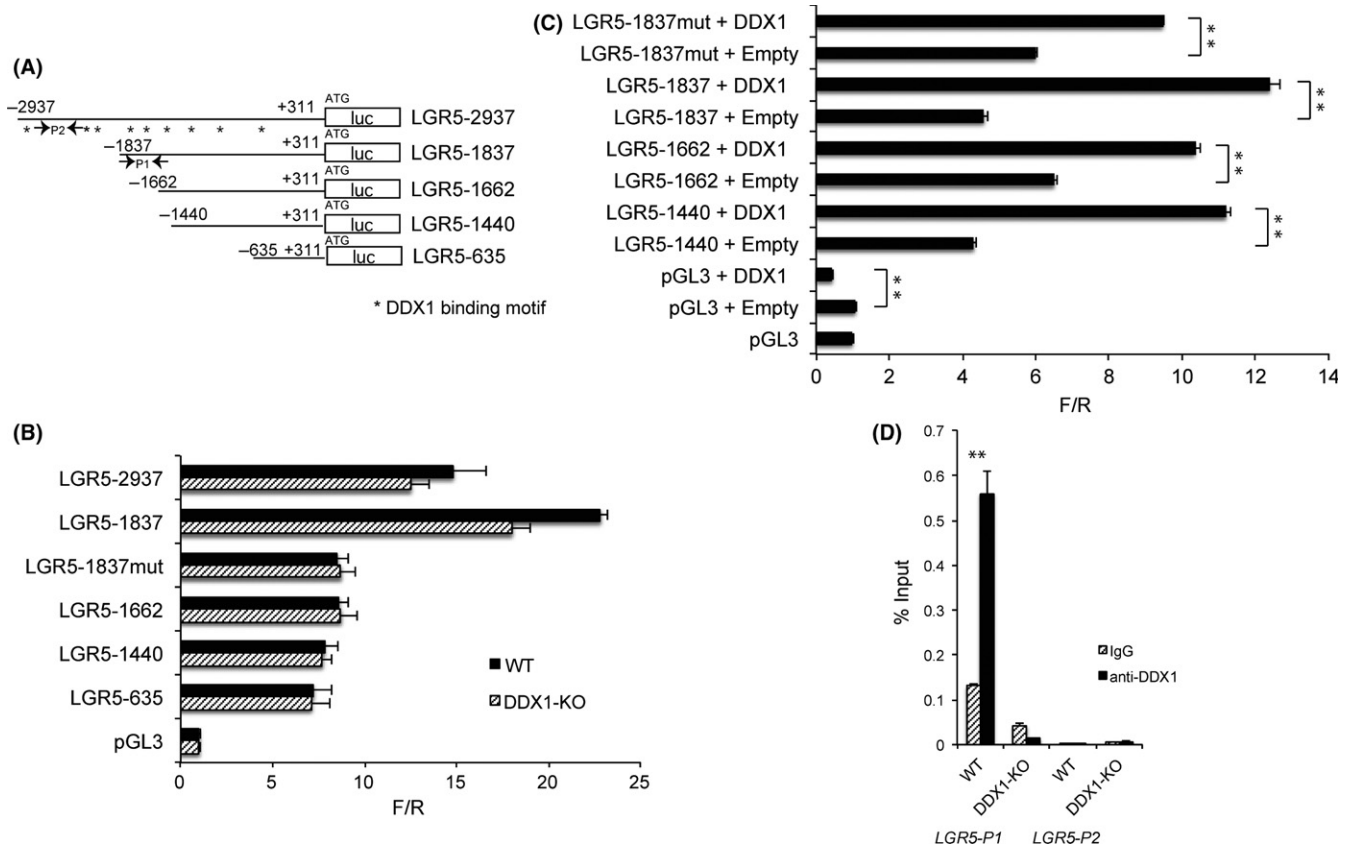


FIGURE 6 Interaction of DDX1 with the enhancer/promoter region of the *LGR5* gene. A, Schematic diagram of reporter constructs. Genomic DNA fragments encompassing −2937 to +311 from the transcriptional initiation site of the human LGR5 gene were inserted into pGL3 vector. B, Luciferase activities of LoVo cell lysates were measured 48 h after transfection, and firefly vs Renilla (F/R) was calculated. C, WT LoVo cells were transfected with the indicated reporter constructs in combination with the DDX1 expression vector (+DDX1) or the empty vector (+Empty). Luciferase activities of cell lysates were measured 48 h after transfection, and firefly vs Renilla (F/R) was calculated. D, WT and DDX1-KO + DDX1 LoVo cells were subjected to ChIP assays using anti-DDX1 or control rabbit IgG. Relative abundance of the *LGR5* gene-specific PCR product is shown. B-D, Each value represents the mean (n = 5) ± SD. $^{**}P < .01$

between −2937 and −1837, and 2 sites between −1837 and −1662 (Figure 6A). Importantly, mutation of AAGGGTGTG (−1680 and −1672) to TTGGGACTG in the LGR5-1837 reporter abrogated the significant activity difference between WT and DDX1-KO LoVo cells (Figure 6B). Next, we transiently transfected the LGR5-1837, 1837 mutant, 1662, 1440 or basic pGL3 reporter into WT LoVo cells in combination with the DDX1 expression vector or the empty vector. F/R values of all the LGR5 reporter-transfected cells were significantly increased by DDX1 overexpression (Figure 6C). Consistent with the data for DDX1-KO cells (Figure 6B), the LGR5-1837 mutant exhibited less F/R value than LGR5-1837, and the DDX1/Empty ratio of the latter was higher than that of the former (Figure 6C). These data suggested a positive action of DDX1 on the −1837 to −1662 region of the human *LGR5* gene. In addition, increases in LGR5-1837 mutant, 1662 and 1440 reporter activities by exogenous DDX1 indicated that 4 other potential DDX1-binding sites between −1662 and +311 could be involved in the transcriptional activation of *LGR5* gene when DDX1 is abundantly present.

To confirm that DDX1 directly interacts with the *LGR5* gene in LoVo cells, we next performed ChIP assays using an anti-DDX1 antibody. We detected specific binding of DDX1 to the −1837 to −1662 region of the *LGR5* gene (Figure 6D). By contrast, a chromatin DNA fragment containing the −2937 to −1837 region was not immunoprecipitated by the DDX1 antibody. These results indicate that DDX1 interacts with the −1837 to −1662 region and directly promotes transcription of the *LGR5* gene in LoVo cells.

4 | DISCUSSION

Here, we propose a critical function for DDX1 in colorectal carcinogenesis using the LoVo cell line as a model. We first showed that *DDX1* mRNA is abundantly expressed in several human colorectal cancer cell lines. Previously, we reported the elevated expression of DDX1 in both seminoma and nonseminoma types of human testicular germ cell tumors. It is of note that *Ddx1* is more abundantly expressed in spermatogonial stem cells than in differentiated spermatocytes in mouse testis.[20] Amplification and/or overexpression of the *DDX1* gene has been described in retinoblastoma,[22] neuroblastoma,[24] glioblastoma[23] and breast cancer.[21] Furthermore, according

to the Catalogue of Somatic Mutations in Cancer (COSMIC, Sanger Institute), certain percentages of cancer tissues are DDX1-positive: 12.6% of liver-derived, 14.7% of lung-derived, 16.9% of ovary-derived, 12.5% of cervix-derived, 7.9% of colorectal-derived, 7.7% of breast-derived and 6.5% of kidney-derived carcinomas. Although point mutations and copy number variations of the *DDX1* gene were barely reported in any cases, the expression level of *DDX1* could be positively correlated with carcinogenesis.

Next, we disrupted the *DDX1* gene in LoVo cells using CRISPR/Cas9 technology. DDX1-KO LoVo cells displayed significantly less proliferation, anchorage-independent colony formation and in vivo tumorigenesis than WT LoVo cells. These phenotypic changes were rescued by exogenous DDX1. These results indicate that DDX1 controls the tumorigenic capacity of colorectal cancer cells.

In DDX1-KO LoVo cells, expression of *LGR5*, *CD133*, *ALDH1* and *SOX2* genes was suppressed. These are colorectal CSC markers.[26-30] Among them, LGR5 expression was fully restored by a high level of exogenously expressed DDX1. Expression of *LGR5* was also repressed by DDX1 knockdown in Colo320 and SW837 cell lines. Subsequently, we found that DDX1 directly binds to the enhancer/promoter region of the human *LGR5* gene and promotes its transcription. As is the case with DDX1, LGR5 is known to be required for proliferation, colony formation and tumor formation of colorectal cancer cells. Disruption of the *LGR5* gene in colorectal and other cancer cell lines results in the loss of tumorigenicity.[9,31-33]

Importantly, LGR5-positive cells act as intestinal stem cells as well as CSC in colon.[10,34] Sphere-forming capacity is one of the reliable properties of CSC. We showed that DDX1-KO LoVo cells produced fewer and smaller spheres than WT and DDX1-KO + DDX1 LoVo cells. LGR5 knockdown CSC display lower proliferation and sphere formation capacity.[35,36] Expression levels of DDX1 in colorectal tumor sections were not positively correlated with histological grade. Consistently, levels of LGR5 in primary colorectal cancers are not correlated with clinicopathological features.[37-39] Expression of LGR5 increases at early stages of colorectal tumorigenesis, and LGR5-positive cells are localized in the crypt base and invasive front areas.[37-39] Taken together, we propose that DDX1 regulates tumorigenic capacity and malignancy of colon cancer cells through the transcriptional regulation of the *LGR5* gene in CSC.

The transcription factor GATA6 enhances the expression of *LGR5* in colorectal cancer cells by direct binding to its enhancer/promoter region.[40] In addition, GATA6 knockdown abrogates the tumorigenic capacity of colorectal cancer cells. In the enhancer/promoter region of the *LGR5* gene, binding sites for GATA6 and DDX1 were distinct, suggesting that DDX1 is a second critical regulator of the *LGR5* gene in colorectal cancer cells.

In summary, DDX1 plays important roles in in vitro colony formation and in vivo tumorigenesis of the human colorectal cancer cell line LoVo. DDX1 is involved in the transcriptional control of the *LGR5* gene, which is a functionally important CSC marker. Therefore, the DDX1-LGR5 axis is a new therapeutic target for colorectal cancers.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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