IOX1 Suppresses Wnt Target Gene Transcription and Colorectal Cancer Tumorigenesis through Inhibition of KDM3 Histone Demethylases

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ABSTRACT

Epigenetic activation of Wnt/β-catenin signaling plays a critical role in Wnt-induced tumorigenesis, notably in colorectal cancers. KDM3 and KDM4 histone demethylases have been reported to promote oncogenic Wnt signaling through demethylation of H3K9 on Wnt target gene promoters and are suggested to be potential therapeutic targets. However, potent inhibitors for these regulators are still not available. In addition, which family is most responsible for activation of Wnt target genes and Wnt-induced oncogenesis is not well documented, specifically in colorectal cancer. In this study, we characterized the functional redundancy and differences between KDM3 and KDM4 in regard to regulating Wnt signaling. Our data suggest that KDM3 may play a more essential role than KDM4 in regulating oncogenic Wnt signaling in human colorectal cancer. We also identified that IOX1, a known histone demethylase inhibitor, significantly suppresses Wnt target gene transcription and colorectal cancer tumorigenesis. Mechanistically, IOX1 inhibits the enzymatic activity of KDM3 by binding to the Jumonji C domain and thereby preventing the demethylation of H3K9 on Wnt target gene promoters. Taken together, our data not only identified the critical mechanisms by which IOX1 suppressed Wnt/β-catenin signaling and colorectal cancer tumorigenesis through inhibition of KDM3, but also suggested that IOX1 may represent an attractive small molecule lead for future drug design and discovery.

Introduction

Human colorectal cancer is the third deadliest cancer worldwide (1). Over 90% of cases are associated with hyperactivated Wnt/β-catenin signaling primarily resulting from adenomatous polyposis coli (*APC*) and β-catenin (*CTNNB1)* mutations (2, 3). When the Wnt pathway is activated, the degradation complex of β-catenin is disrupted, allowing β-catenin to accumulate and be transported into the nucleus to bind to T-cell factors (TCF; REF. 4). β-Catenin competes against Wnt gene transcriptional corepressors, such as Groucho and CtBP, for TCF binding. Once bound, β-catenin can recruit Wnt gene transcriptional co-activators, epigenetic regulators, and chromatin remodeling complexes. These factors, including CBP/P300, BCL9/PYGO complex, polymerase-associated factor 1, SET1, and KDM3/4 family histone demethylases, are all reported to directly interact with β-catenin and play critical roles during transcriptional activation (4–8). The importance of Wnt/β-catenin signaling in colorectal cancer initiation and development, and validation of the Wnt/β-catenin pathway as a therapeutic target for colorectal cancer treatment has already been demonstrated by numerous studies (9, 10). Unfortunately, no FDA-approved Wnt inhibitor has been successfully developed. Thus, an unmet need still exists to develop inhibitors of Wnt/β-catenin signaling for successful eradication of colorectal cancer.

Emerging evidence suggests that epigenetic regulation of Wnt/β-catenin signaling plays a pivotal role in tumorigenesis. Previously, we have identified that KDM3A and KDM3B histone demethylases epigenetically control tumorigenic potential of cancer stem cells (CSC) in human colorectal cancer through Wnt signaling (11). These histone demethylases are recruited by β-catenin to Wnt target gene promoters for H3K9me2 demethylation, thereby releasing the transcriptional suppression. Simultaneously, KDM3 also recruits histone methyltransferase MLL1 to the promoter region, which adds a methyl group to H3K4, thus synergistically activating Wnt target gene transcription. In comparison with the KDM3 family, the KDM4 family members, KDM4A, KDM4B, and KDM4C, which demethylate H3K9me2/me3, have also been reported to associate with β-catenin to positively regulate Wnt-induced oncogenesis in multiple types of tumor, including colorectal cancer (12–16). KDM4D is also enzymatically active, but it lacks the double PHD and Tudor domains that are involved in recognizing methylated histone residues and has a different substrate specificity than KDM4A/B/C (12, 17). Although these regulators activate Wnt target genes transcription through a similar epigenetic mechanism, the functional redundancy between these family members in regulating Wnt signaling has not been well characterized. In addition, determination of which family is most responsible for activation of Wnt target genes and Wnt-induced tumorigenesis, especially in colorectal cancers, is not documented. In this study, we revealed that KDM3 may play a more essential role than KDM4 in regulating oncogenic Wnt signaling in human colorectal cancer through characterization of the functional redundancy and differences between the two families. This suggests that targeting KDM3 may represent a promising therapeutic approach for colorectal cancer

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Mol Cancer Ther 2021;20:191–202

doi: 10.1158/1535-7163.MCT-20-0328

treatment. Therefore, a small-molecule inhibitor, IOX1, was selected to investigate the viability of targeting KDM3 in colorectal cancer.

IOX1 is a known broad-spectrum inhibitor of 2-oxoglutarate oxygenases, and it has a strong inhibitory effect against KDM3 and KDM4 (18, 19). In addition, a previous report showed it having the lowest IC_{50} value against KDM3A (18). Studies have demonstrated that IOX1 presents antiproliferative effects on HeLa cells, hepatocellular carcinoma, and β-thalassemia cells, but whether it can suppress colorectal cancer tumorigenesis has not been investigated (19–21). We found that IOX1 may suppress Wnt target gene transcription and colorectal cancer tumorigenesis primarily through inhibition of KDM3. Collectively, our results demonstrate that KDM3 is more decisive than KDM4 in epigenetic control of Wnt signaling and colorectal cancer tumorigenesis and suggest that further drug design and discovery of KMD3 inhibitors by adopting IOX1 as a lead may be beneficial for colorectal cancer treatment.

Materials and Methods

Chemicals

IOX1 (#14056) was obtained from Active Motif. Tween 80 (#MP021947251) and dimethyl sulfoxide (#BP231) were obtained from Fisher Scientific.

Cell culture

293T, HCT116, and DLD-1 cells were obtained from the ATCC and maintained in DMEM medium containing 10% FBS and antibiotics. HCP-1 cells were obtained from MD Anderson Cancer Center (Huston, TX). All cell lines were authenticated by short tandem repeat genotyping and tested to be *Mycoplasma* free. To generate the HCT116-TCF-Luc stable cells, pGreenFire1-TCF/LEF (EF1α-puro) Lentivector and packaging pPACK-H1 Lentiviral Packaging Plasmid Mix were obtained from System Bioscience. For viral particles production, lentivirus was produced by co-transfection of pGreenFir1-TCF/LEF1 with packaging plasmids into 293T cells. HCT116 cells were incubated with viral particles for 12 hours. Two days post virus infection, the cells were selected using puromycin (1 mg/mL) for 4 days.

Oligonucleotides and antibodies

The siRNA oligonucleotide sequences were: *KDM3A* (5′-GGAGAAGATTTTAGAGATA-3′ and 5′-GCATAGGACTGGCATTATA-3′); *KDM3B* (5′-CCGAAAGGUUGGAGAAGAA-3′ and 5′-GCTGAAAGATGTAAGCAAA-3′); *KDM4A* (5′-GCTGCAGTATTGAGATGCTAA-3′ and 5′-GCACCGAGTTTGTCTTGAAATT-3′); *KDM4B* (5′-GCCTCTTCACGCAGTACAA-3′ and 5′-GGACTTTGATGACCTTGAA-3′); *KDM4C* (5′-CATCAGTGGCAGAGAGTAA-3′ and 5′-CCTAAGGAGTGGAAGCCAA-3′); and *CTNNB1* (5′-CCTTTAGCTGTATTGTCTGAA-3′ and 5′-AGGTGCTATCTGTCTGCTCTA-3′). Antibodies used were: anti-KDM3A (Bethyl; #A301–539A), anti-KDM3B (Bethyl; #A300–883A), anti-KDM4A (Bethyl; #A300–861A), anti-KDM4B (Bethyl; #A301–478A), anti-KDM4C (Bethyl; #A300–885A), anti-H3K9me2 (Abcam; #ab1220), anti-H3K9me3 (Abcam; #ab8898) and anti-α-Tubulin (Sigma-Aldrich; #T9026). The primer sequences used for qPCR are listed in Supplementary Tables S2 and S3.

siRNA transfection and luciferase assays

For transient knockdown of KDM3, KDM4 or β-catenin, HCT116 or DLD-1 cells were plated at 40% to 50% confluence in standard 12-well plates and incubated overnight. The cells were then transfected with various amounts of siRNA using Lipofectamine RNAiMAX (Life Technologies) following the manufacturer's instructions. For luciferase assays in HCT116-TCF-Luc cells, 48 hours post siRNA transfection or 12 hours post IOX1 treatment, the luciferase activity of total cell lysates was measured using Bright-Glo Luciferase Assay System (Promega). The reporter activity was normalized against the protein concentration of each cell lysate sample.

RNA-seq

Library preparation and RNA-sequencing (RNA-seq) were performed via GeneWiz RNA-seq service. Alignment of reads was done using Tophat with the hg18 build of the human genome (https://ccb.jhu.edu/software/tophat/index.shtml). Transcript assembly and differential expression were determined using Cufflinks with Refseq mRNAs. The analysis of RNA-seq data was performed using the cummeRbund package in R (http://cole-trapnell-lab.github.io/cufflinks/). The heatmap was generated with Heatmap Builder (http://ashleylab.stanford.edu/tools/tools-scripts.html). GSEA and the statistical analyses were performed with GSEA software (http://www.broad.mit.edu/GSEA) and a two-tailed *t* test, respectively.

ChIP-qPCR analysis

For each chromatin immunoprecipitation (ChIP) reaction mixture, 10^6 cells were used. HCT116 or HCP-1 cells were treated with 10 mmol/L dimethyl 3,30-dithiobispropionimidate-HCl (DTBP; Thermo Fisher #20665) in PBS at room temperature for 10 minutes and then fixed with 1% formaldehyde at 37°C for 10 minutes. Total cell lysates were sonicated to generate 400–600 bp DNA fragments. Chromatin complexes were immunoprecipitated with the antibodies as indicated. The precipitated DNA samples were measured by qPCR. Data are expressed as the percentage of input DNA.

Computational modeling

Currently, the crystal structures of KDM3B, KDM4B, and KDM4C have been determined and reported (22–24). According to the amino acid sequence, the amino acid sequence identity between KDM3A and KDM3B is about 60%. Thus, the crystal structure of KDM3B (PDB ID: 4C8D) was used as the template protein to build the 3D conformations of KDM3A (23). Before homology modeling, sequence alignment was conducted between the target and template amino acid sequences. The amino acid sequences of the JmjC domain of KDM3A (Q9Y4C1) and KDM3B (Q7LBC6) were downloaded from UniProt (25). Multiple sequence alignments were conducted using the program ClustalX 2.1 with default parameters (26). According to these sequence alignments, homology models of the human KDM3A were built by Swiss-model.

In addition, the crystal structure of KDM4A complexed with IOX1 was determined in 2010 (27). Moreover, the active site of the crystal structure of KDM3B also accommodated a small molecule, *N*-oxalylglycine (23). The two crystal structures indicated that IOX1 was positioned in a similar location of the active site in KDM4A to that of *N*-oxalylglycine in KDM3B. As KDM4A, KDM4B, and KDM4C shared about 80% amino acid sequence identities, the binding modes of IOX1 with KDM4B and KDM4C were almost the same as that of IOX1 with KDM4A. Therefore, according to the ligand–receptor interactions between *N*-oxalylglycine and KDM3B, between IOX1 and KDM4A (23, 27), IOX1 was docked into KDM3B, KDM4B, KDM4C, and the homology model of KDM3A. The docking poses with the highest CHEM-PLP score were selected as the optimal binding modes to conduct the following analyses.

CCK-8, clonogenic formation assay, wound healing, and invasion assay

Cell proliferation was measured using the Cell Counting Kit-8 (MedChemExpress; # HY-K0301) following the manufacturer's instruction. For the clonogenic assay, cells were seeded at 200 cells/well until attached to well for 4 hours and followed by IOX1 treatment. Cells were incubated until they grew to approximately 50 colonies with fresh media added every 2 days. The cells were stained using 0.5% crystal violet and imaged with a camera. Data were collected via ImageJ analysis. Wound healing and invasion assays were conducted as previously described (28).

Flow cytometry and sphere formation assay

For flow cytometry analysis, the cell suspension of HCT116 or HCP-1 cells was filtered through a 40-μm mesh filter. Subsequently, tumor cells were stained with the ALDHEFLUOR assay Kit (STEMCELL Technologies; #01700) following the manufacturer's guidelines to label the $ALDH^{high}$ populations. The ALDEFLUOR-stained cells were treated with diethylaminobenzaldehyde, a specific ALDH inhibitor that served as ALDH-negative controls. The $ALDH^{high}$ populations were determined by FCSExpress 7 (De Novo Software). Tumor sphere formation assays were as performed as previously described (28).

In vivo tumor growth

A total of 5×10^3 FACS-sorted cells $ALDH^{high}$-HCP-1 cells were mixed with Matrigel and injected subcutaneously into both left and right flank of the nude mice. The mice were randomly divided into three groups after inoculation ($n = 8$ per group). The treatment group was intraperitoneally injected with IOX1 or DMOG 10 mg/kg daily and the control group was injected with only vehicle for 17 days. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the Virginia Commonwealth University.

IHC

IHC assays were performed using the Vectastain Elite ABC Kit (Vector, #PK-6101) following the manufacturer's guidelines as describe previously (28). The antibodies used in IHC assays were anti-AXIN2 (Abcam, #ab109307) and anti-CCND1 (Abcam, #ab134175).

Data availability

The accession number for the RNA-seq data reported in this article is NCBI GEO: GSE149233.

Results

KDM3 plays a more essential role than KDM4 in regulating Wnt target gene transcription in human colorectal cancer cells

It is well-known that Wnt/β-catenin signaling plays an important role in human colorectal cancer initiation and development. To characterize the functional difference between KDM3 and KDM4 in regulating Wnt signaling in colorectal cancer, we used human colorectal cancer HCT116 cells stably expressing Wnt/TCF-responsive luciferase reporter (HCT116-TCF-Luc) to measure β-catenin/TCF-mediated transcription. HCT116 cells have constitutively active β-catenin/TCF-mediated transcription due to Ser45 mutation in β-catenin. Knockdown of KDM3 and KDM4 family demethylases individually by siRNA significantly suppressed β-catenin/TCF-mediated transcription in HCT116-TCF-Luc cells by a 40% to 70% reduction of the luciferase activity (**Fig. 1A**). Of note, triple knockdown of KDM4A/B/C simultaneously had a similar inhibitory effect as compared with the individual knockdowns. In contrast, double knockdown of KDM3A and KDM3B simultaneously had a more dramatic inhibitory effect compared with individual knockdowns resulting in over 90% inhibition of the luciferase activity, similar to β-catenin depletion (**Fig. 1A**). Efficient knockdowns of KDM3A, KDM3B, KDM4A, KDM4B, and KDM4C, individually or simultaneously were confirmed by western blots (**Fig. 1B**). Taken together, these results suggest that the KDM3 family demethylases may play a more critical role than KDM4 family in regulating β-catenin/TCF-mediated transcription in colorectal cancer cells. Our results also suggest that there is a functional redundancy between the KDM3 family members, which coincides with our previous studies (11). Therefore, KDM3A/B were simultaneously knocked down in the remainder of the studies.

To further confirm our results, we examined well-characterized endogenous Wnt target genes, *AXIN2* and *DKK1*, as well as the well-known key oncogene, *CCND1*, expression levels after depletion of KDM3 or KDM4 members in HCT116 cells by using quantitative real-time reverse transcriptase PCR (RT-qPCR; **Fig. 1C**). Our previous study indicated that KDM3 maintains CSC-like properties through Wnt signaling in human colorectal cancer (11). Therefore, we also examined several colorectal CSC markers that are also Wnt direct targets: *ASCL2, RNF43, LGR5*, and *ZNRF3*. In addition, similar experiments were also performed in another human colorectal cancer cell line, DLD-1, which contains an *APC* mutation. Efficient knockdowns of KDM3/4 in DLD-1 cells were also confirmed by western blots (Supplementary Fig. S1). Depletion of KDM3 showed a stronger inhibitory effect than depletion of KDM4. As shown in **Fig. 1D**, depletion of KDM4 family demethylases individually or simultaneously only suppressed a subset of Wnt target genes transcription, such as *AXIN2, DKK1*, and *ASCL2*. In comparison, the Wnt target genes we examined were all significantly suppressed by knockdown of KDM3A and KDM3B simultaneously. Of note, *LGR5* is not expressed at detectable levels in HCT116 cells, and *DKK1* is not expressed at detectable levels in DLD-1 cells. Taken together, these results suggest that the KDM3 family may play a more essential role than KDM4 in regulating Wnt target gene transcription in colorectal cancer.

IOXI suppresses Wnt signaling in colorectal cancer cells

IOX1 is a known broad-spectrum inhibitor of histone demethylases, which includes both KDM3 and KDM4 (19). Therefore, we speculated that IOX1 may suppress Wnt target gene transcription through inhibiting the enzymatic activities of KDM3 and KDM4. To test our hypothesis, we first used the luciferase reporter assay to detect whether IOX1 could suppress β-catenin/TCF-mediated transcription in HCT116-TCF-Luc cells. As shown in **Fig. 2A**, IOX1 significantly suppressed luciferase activity in HCT116-TCF-Luc cells in a dose-dependent manner. This suggested that IOX1 could potentially inhibit Wnt signaling.

To examine how IOX1 affects the global Wnt target gene transcriptome in colorectal cancer cells, we performed RNA-seq in HCT116, DLD-1, and HCP-1 cells treated with IOX1. HCP-1 cells were from freshly isolated human colorectal cancer cells (29). The gene set enrichment analysis (GSEA) revealed that the expression of Wnt/β-catenin target genes in colorectal cancer cells was significantly downregulated after IOX1 treatment in all the above mentioned cells (**Fig. 2B**; Supplementary Fig. S2A–S2C; ref. 30). Furthermore, GSEA showed that Wnt signature genes in colorectal cancer adenoma and carcinoma were also downregulated after IOX1 treatment (**Fig. 2C** and **D**; Supplementary Fig. S2A, S2B, and S2D; ref. 31). In addition, we examined whether IOX1 treatment can suppress CSC signature genes. GSEA indicated that the cancer stemness signatures (32), as well as

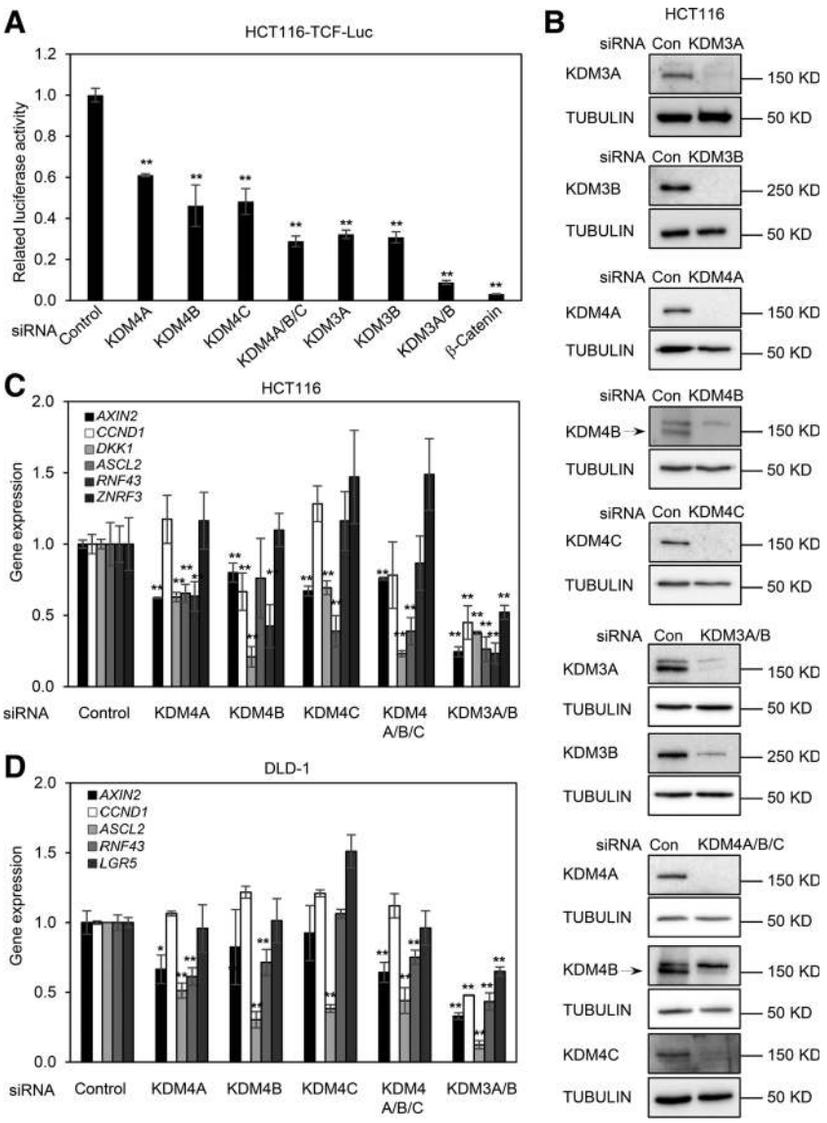

Figure 1.

KDM3 plays a more critical role than KDM4 in regulating Wnt target gene transcription in human colorectal cancer cells. **A,** Analysis of luciferase activity of HCT116-TCF-Luc cells treated with the indicated siRNA relative to those transfected with control siRNA. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student t test ($n = 3$). **B,** KDM3A, KDM3B, KDM4A, KDM4B, and KDM4C were knocked down by siRNA in HCT116 cells. **C** and **D,** The knockdown of KDM3A and KDM4 inhibited the expression of Wnt target genes in HCT116 (**C**) and DLD-1 (**D**) cells by real-time RT-PCR. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student t test ($n = 3$).

Wnt signatures in intestinal stem cells (ISC; ref. 33), which represent the origin of colorectal cancer, were significantly downregulated after IOX1 treatment (**Fig. 2E** and **F**; Supplementary Fig. S2A and S2B).

RT-qPCR also confirmed that IOX1 profoundly inhibited the expression of Wnt target genes *AXIN2, DKK1*, and *CCND1* in HCT116, DLD-1, and HCP-1 cells in a dose-dependent manner (**Fig. 2G**). In addition, the colorectal CSC signature genes, such as *ASCL2, LGR5, RNF43*, and *ZNRF3*, were significantly suppressed by IOX1 treatment (**Fig. 2G**). Of note, most of these Wnt target genes could be inhibited by 50% at 100 μmol/L concentration of IOX1 (**Fig. 2G**). Thus, our results indicated that IOX1 can significantly suppress Wnt target gene transcription in human colorectal cancer cells.

IOX1 predominately inhibits Wnt target gene transcription through KDM3

Previous studies indicated that KDM3 can directly associate with β-catenin and bind to Wnt target gene promoters to activate transcription through demethylation of H3K9me2 (11). In addition to KDM3, KDM4 has also been reported to regulate Wnt target gene transcription through demethylation of H3K9me3/me2 (13–15, 34). To investigate the potential molecular mechanism of IOX1 in suppressing Wnt target gene transcription, we performed ChIP, followed by qPCR to examine the enrichments of KDM3, KDM4, and H3K9me3/me2 on *AXIN2* and *DKK1* promoters in HCT116 cells. As a strict negative control (NEG), a region located 10kb downstream of the transcription terminal site of *AXIN2* and/or *DKK1*, was also examined. As shown in **Fig. 3A** and **B**, the recruitment of all family members of KDM3 and KDM4 to these regions, especially at the Wnt-responsive element (WRE), was not affected upon IOX1 treatment. Similar findings were also observed at the WRE regions of the *AXIN2* and *DKK1* promoters in HCP-1 cells (Supplementary Fig. S3A and S3B). Notably, IOX1 treatment resulted in enhancement of both H3K9me3 and H3K9me2 enrichments at the WRE, but not at NEG or 45S rDNA, an alternative negative control for examining the enrichment of H3K9me3/2, suggesting that demethylation of H3K9 on Wnt target gene promoters can be inhibited by IOX1 (**Fig. 3C** and **D**, Supplementary Fig. S3C and S3D). In particular, the

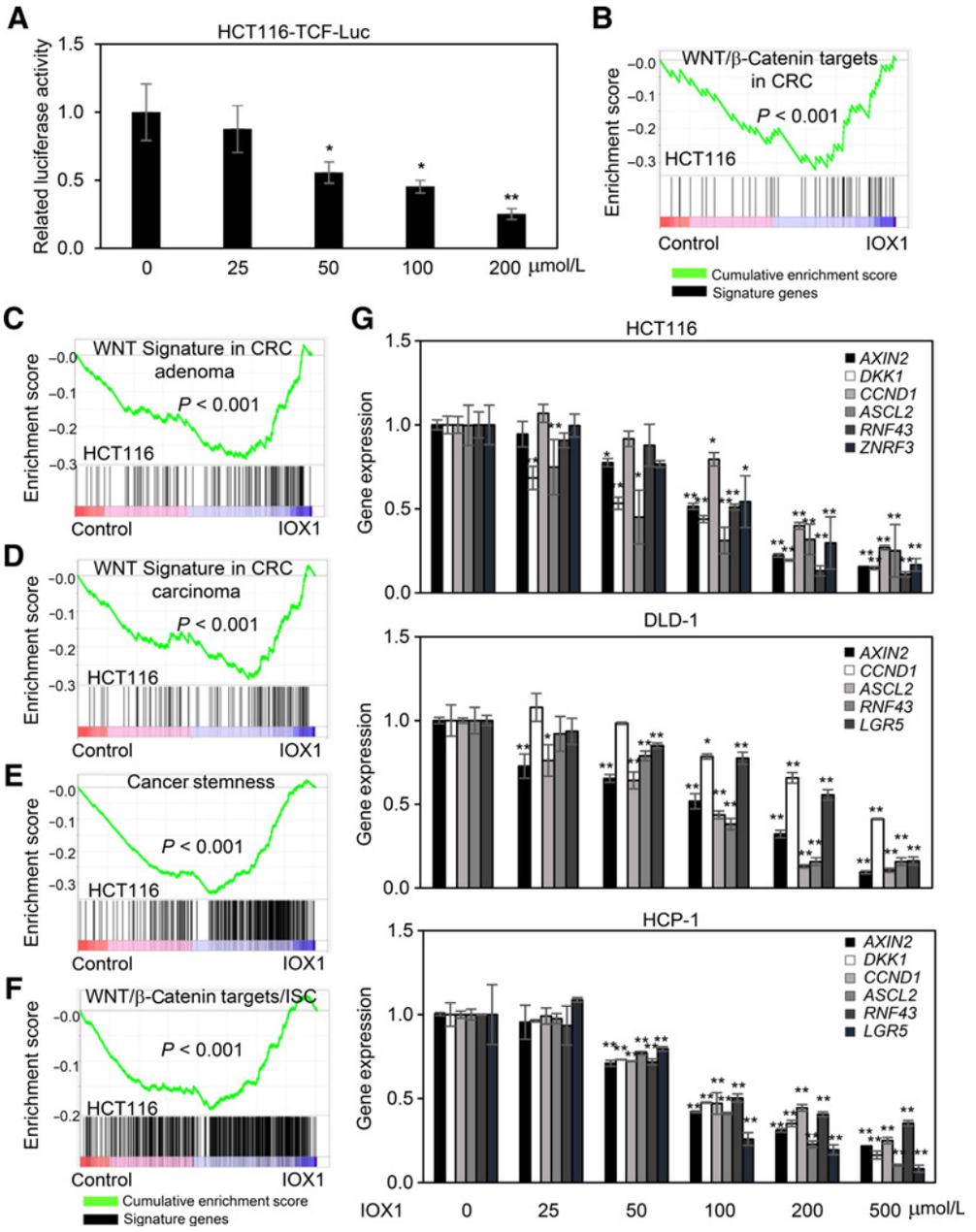

Figure 2.

IOX1 suppresses Wnt/β-catenin signaling in colorectal cancer cells. **A,** IOX1 inhibited Wnt reporter activities in HCT116-TCF-Luc cells. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student t test ($n = 3$). **B–F,** Quantitative comparison of genes downregulated in HCT116 cells by IOX1 treatment (100 μmol/L) and Wnt/β-catenin target gene signatures as indicated using GSEA. $P < 0.001$. **G,** IOX1 inhibited the expression of Wnt target genes in HCT116, DLD-1, and HCP-1 cells. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student t test ($n = 3$).

enhanced enrichment of H3K9me2 was more dramatic than H3K9me3. This suggests that IOX1 may exert its inhibitory effect on Wnt target gene transcription primarily through suppressing H3K9me2 demethylation.

Because IOX1 mostly affects H3K9me2 demethylation, and considering KDM3 plays a more critical role than KDM4 in regulating Wnt signaling in colorectal cancer, we hypothesize that IOX1 may exert its function in suppressing Wnt target transcription predominatly through suppressing KDM3. To test this, we knocked down KDM3A/B or KDM4A/B/C expression in HCT116 cells using siRNA and treated the cells with IOX1. As shown in **Fig. 3E**, IOX1 can still dramatically inhibted Wnt target genes' transcription in HCT116 cells after depletion of KDM4A/B/C, including *AXIN2, DKK1, CCND1, RNF43*, and *ZNRF3*. On the contrary, all these aformentioned genes, except *DKK1*, cannot be significatly downregulated by IOX1 after KDM3A/B were depleted. We also obtained similar results in DLD-1

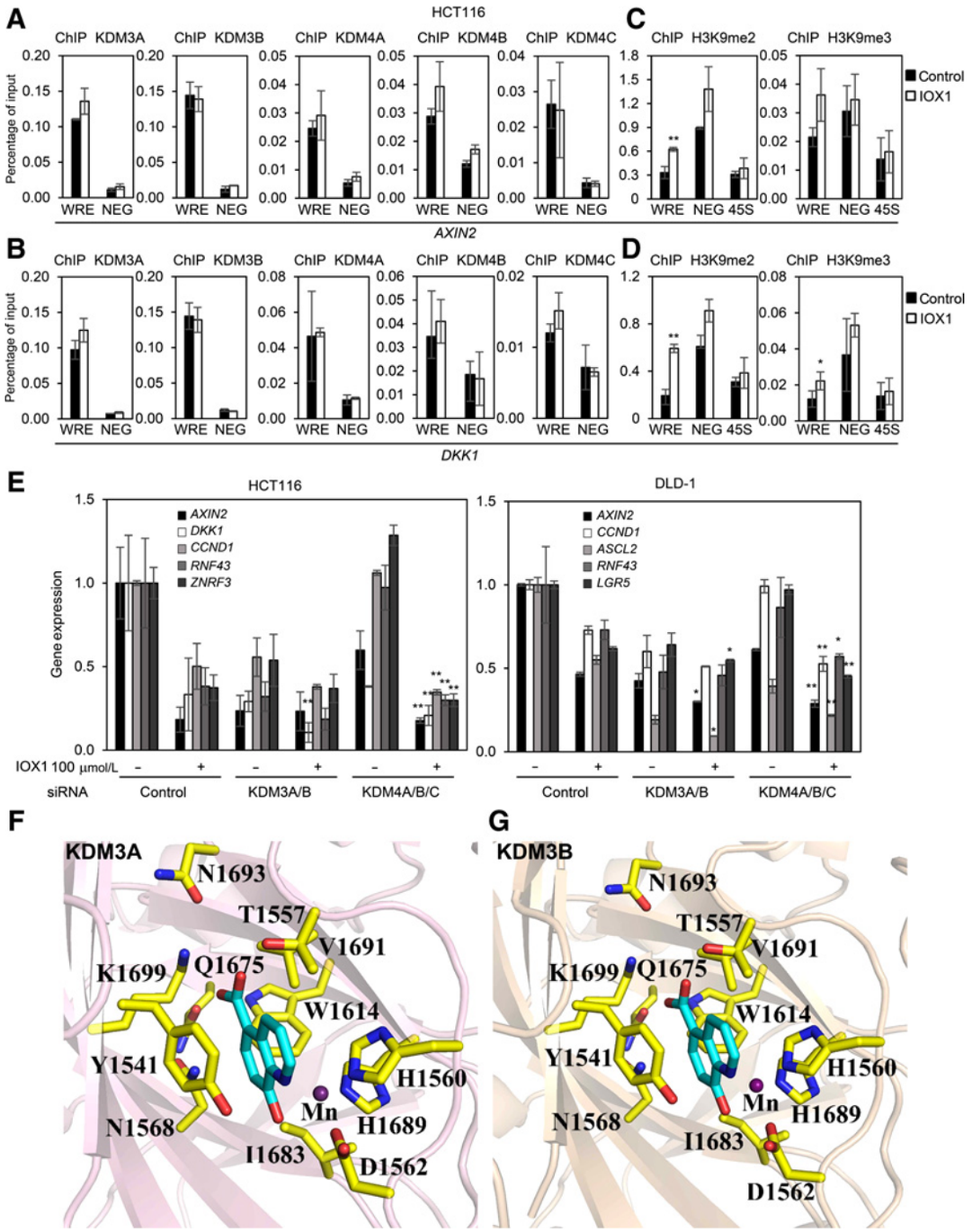

Figure 3.

IOX1 suppresses Wnt target gene transcription primarily through inhibiting KDM3. **A** and **B,** ChIP assays confirmed that the IOX1 did not affect the enrichments of KDM3 and KDM4 on *AXIN2* (**A**) and *DKK1* (**B**) promoters in HCT116 cells. **C** and **D,** IOX1 enhanced the level of H3K9me3/2 on *AXIN2* (**C**) and *DKK1* (**D**) promoters in HCT116 cells. **E,** IOX1 inhibited the expression of Wnt target genes in HCT116 and DLD-1 cells primarily through KDM3. HCT116 and DLD-1 cells were treated with 100 μmol/L of IOX1 and siRNA as indicated. **F** and **G,** The binding modes of IOX1 with KDM3A (**F**) and KDM3B (**G**). KDM3A and KDM3B are shown as cartoon models in light pink and wheat, respectively. IOX1 and key amino acid residues in KDM3 family are shown as stick models. Carbon atoms, IOX1 in cyan; key amino acid residues of KDM3 family in yellow. The metal ion (Mn) of KDM3 family shown as sphere models in purple. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$, unpaired two-tailed Student t test ($n = 3$).

cells (**Fig. 3E**). Taken together, these results strengthened our hypothesis that IOX1 suppressed Wnt target gene transcription primarly through KDM3.

To rationalize the recognition of IOX1 to KDM3A and KDM3B, molecular docking studies were conducted. Multiple sequence alignment of KDM3A and KDM3B proteins showed that these proteins share about 79% homology and 60% identity (Supplymentary Supplementary Fig. S3F). It was observed that both proteins share a highly conserved binding pocket. The crystal structure of KDM3B in complex with a small molecule, *N*-oxalylglycine, has been reported (PDB ID 4C8D; ref. 23). Based upon the sequence alignments in Supplementary Fig. S3F, a homology model of human KDM3A was constructed through a Swiss-model (35). It was apparent that the 3D conformation of KDM3A almost mimicked that of its template protein, KDM3B (Supplementary Fig. S3G). Docking studies of IOX1 were then carried out using GOLD5.6 where the binding pocket was defined on the basis of the co-crystalized ligand *N*-oxalylglycine in KDM3B structure and the binding pose of IOX1 with the highest CHEM-PLP score was selected as the optimal binding mode. It was observed that IOX1 showed almost identical interactions with both KDM3A and KDM3B, which included hydrogen-bonding interactions, metal-coordinating interactions (with Mn), and hydrophobic interactions (**Fig. 3F** and **G**). Taken together, these models provide a plausible explanation for molecular mechanism of IOX1 and how it may inhibit histone demethylase activity of KDM3.

Similar molecular-docking studies were conducted between the KDM4 family members and IOX1 as well. KDM4A, KDM4B, and KDM4C shared about 80% amino acid sequence identities (Supplemenatry Supplementary Fig. S3H). The crystal structures of KDM4B and KDM4C were already available. In addition, the crystal structure of KDM4A complexed with IOX1 has been determined (19, 27). These crystal structures indicated that IOX1 was positioned in an almost identical location of the active site in KDM4A to that of *N*-oxalylglycine in KDM3B with a very similar binding mode. IOX1 was docked into KDM4B and KDM4C with the highest CHEM-PLP score as the optimal binding modes (Supplemenatry Supplementary Fig. S3I and S3J). Yet, four different key amino acid residue interactions were observed in the KDM3A/IOX1, KDM3B/IOX1, KDM4B/IOX1, and KDM4C/IOX1 complexes, which may help understand the differentiated potency and selectivity of IOX1 to these enzymes (Supplementary Table S1). Overall, these docking results showed that IOX1 may interact with the Jumonji C (JmjC) domain that folds into eight-sheets to form an ezymatically active pocket and is essential for demethylase activity. These results help explain how IOX1 inhibits the enzymatic activity of these KDM families in putatively differentiated fashions, and may provide a reasonable explanation for the molecular mechanisms of IOX1 inhibiting histone demethylase activity in the KDM3 and KDM4 families.

IOX1 inhibits cell proliferation, migration, and invasive growth of colorectal cancer *in vitro*

Wnt signaling promotes proliferation and invasive growth of colorectal cancer cells. To examine whether IOX1 suppresses the malignant phenotypes of colorectal cancer cells induced by Wnt signaling, we treated HCT116 and DLD-1 cells with IOX1. As shown in **Fig. 4A** and **B**, IOX1 treatment led to a significant decrease of cell proliferation and clonogenic activities of both HCT116 and DLD-1 cells in a dose-dependent manner. In addition, IOX1 treatment significantly inhibited the motility (**Fig. 4C**) and migration abilities of colorectal cancer cells (**Fig. 4D**). These results suggest that IOX1 can suppress the malignant phenotypes of colorectal cancer cells.

IOX1 inhibits colorectal CSC-like properties through Wnt signaling

Because hyperactivated Wnt activity has been shown to be an important characteristic of CSCs in human colorectal cancer, we wanted to examine whether IOX1 would inhibit CSC-like properties in colorectal cancer. Aldehyde dehydrogenase-high ($ALDH^{high}$) subpopulations in human colorectal cancer cells exhibit CSC-like properties and is a well-characterized marker for isolating CSCs *in vitro* and *in vivo* (36). The cell sorting profiles showed that IOX1 dramatically reduced the $ALDH^{high}$ CSC population of HCT116 and HCP-1 from 5.3% to 2.3% and from 14.5% to 4.1%, respectively (**Fig. 5A**). This suggests that the self-renewal ability of CSCs can be suppressed by IOX1. To further verify our results, we isolated the $ALDH^{high}$ CSCs from HCT116 and HCP-1 cells. As an *in vitro* measure of CSC-like behavior, tumor sphere formation assays were used as a surrogate for CSC-like self-renewal (36). IOX1 abolished the tumor sphere formation abilities of both $ALDH^{high}$-HCT116 and $ALDH^{high}$-HCP-1 CSCs (**Fig. 5B**). Of note, the tumor sphere formation was almost eliminated by IOX1 at 25 μmol/L. Taken together, these results suggest that IOX1 can inhibit the self-renewal ability of colorectal CSCs.

To further confirm the ability of IOX1 to suppress CSC-like properties through Wnt signaling, we conducted RT-qPCR in $ALDH^{high}$-HCT116 and $ALDH^{high}$-HCP-1 cells. The results showed that IOX1 significantly inhibited the expression of Wnt target genes in both CSCs (**Fig. 5C**). Most of these Wnt target genes were inhibited by 50% from 50 μmol/L of IOX1 in sorted CSCs, as compared with the 100 μmol/L treatment in the unsorted cells (**Fig. 2G**). This suggests that IOX1 presents stronger inhibitory effects of Wnt target gene transcription in CSCs as compared with the whole population of culture colorectal cancer cells.

In addition, western blots showed that the expression of both KDM3A and KDM3B was dramatically upregulated in $ALDH^{high}$-HCT116 CSCs as compared with the $ALDH^{low}$-HCT116 non-CSCs, whereas KDM4A, KDM4B, and KDM4C expression remained similar between CSCs and non-CSCs (**Fig. 5D**). Similar findings were also observed by comparing these demethylases' expression levels between $ALDH^{high}$-HCP-1 CSCs and $ALDH^{low}$-HCP-1 non-CSCs using RT-qPCR (**Fig. 5E**). These interesting discoveries further strengthened our conclusion that KDM3 may play a more essential role than KDM4 in Wnt-induced tumorigenesis in colorectal cancer.

IOX1 suppresses tumorigenicity of human colorectal CSCs

To determine whether IOX1 suppressed the tumorigenic potentials of colorectal CSCs *in vivo*, we subcutaneously inoculated the $ALDH^{high}$-HCP-1 cells into nude mice and then treated the mice with IOX1. We also tested the effect of another small-molecule KDM4 inhibitor, DMOG, for its ability to suppress colorectal cancer tumorigenesis in parallel (37). Studies indicate that DMOG can inhibit KDM4 activity and enhance H3K9 methylation level *in vivo* (38, 39). In our studies, IOX1 significantly suppressed both tumor size and tumor weight whereas DMOG had little effect on tumor growth suppression (**Fig. 6A–C**). Importantly, administration of IOX1 had little impact on body weight (**Fig. 6D**). Consistent with inhibition of Wnt signaling, IHC staining also confirmed that IOX1 treatment dramatically reduced

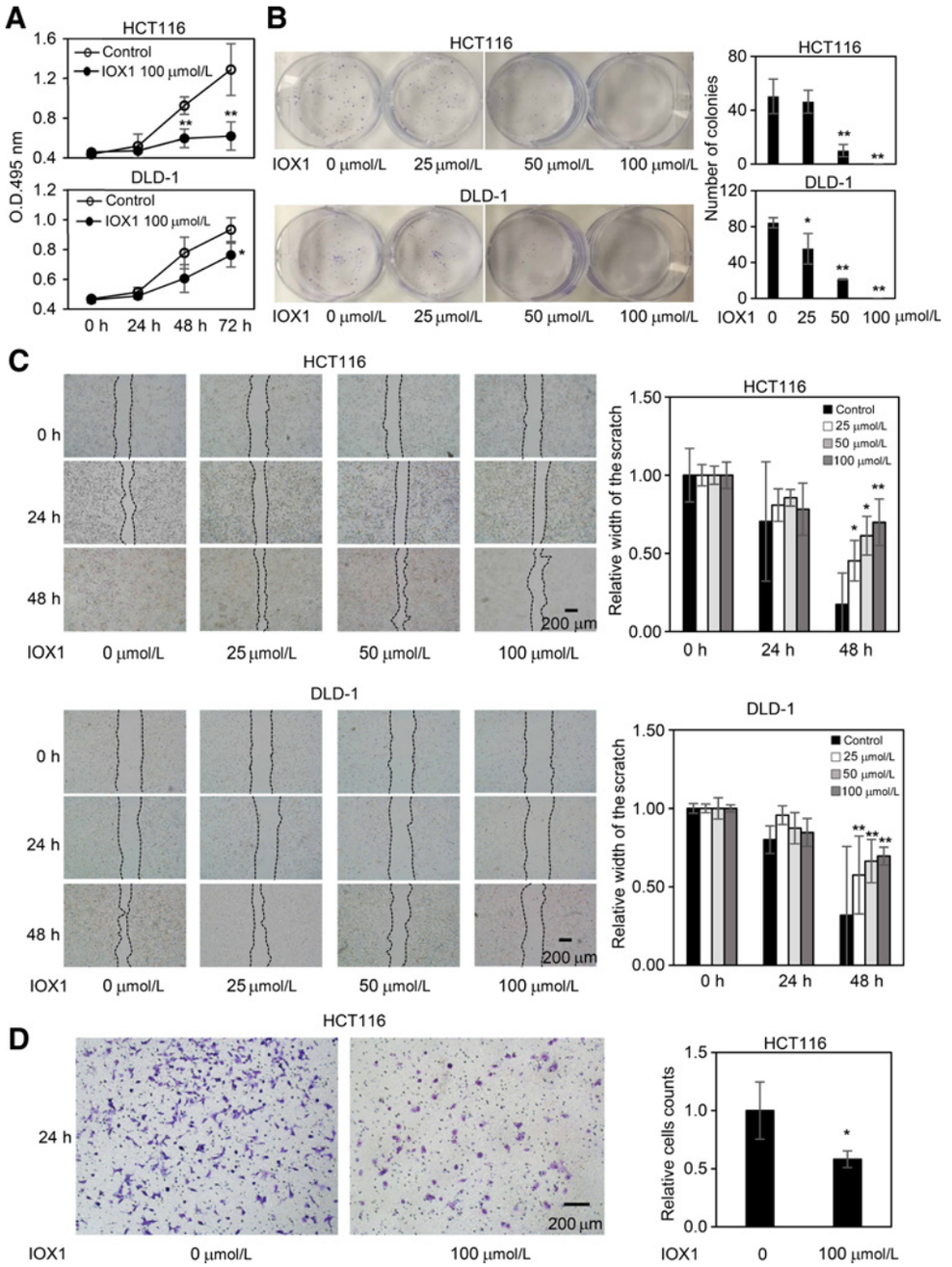

Figure 4.

IOX1 inhibits cell proliferation, migration, and invasive growth of colorectal cancer *in vitro*. **A,** Cell proliferation was measured by CCK8 assay in HCT116 and DLD-1 cells treated with 100 μmol/L of IOX1. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student *t* test ($n = 3$). **B,** IOX1 suppressed tumor growth of colorectal cancer cells by colony formation assay. HCT116 and DLD-1 cells were treated with increasing concentration of IOX1 as indicated. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student *t* test ($n = 3$). **C,** The ability of cell migration and motility was evaluated by wound-healing assay in colorectal cancer cells treated with increasing concentration of IOX1 as indicated. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student *t* test ($n = 3$). **D,** IOX1 inhibited cell migration and invasion of HCT116 cells. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ by Student *t* test ($n = 3$).

Wnt direct targets, *AXIN2* and *CCND1*, expression in xenograft tumors (**Fig. 6E**). Taken together, these results suggest that IOX1 can potently inhibit colorectal cancer tumorigenesis *in vivo*.

Discussion

Epigenetic mechanisms are arising as key players in cancer development, and emerging evidence suggests that epigenetic factors

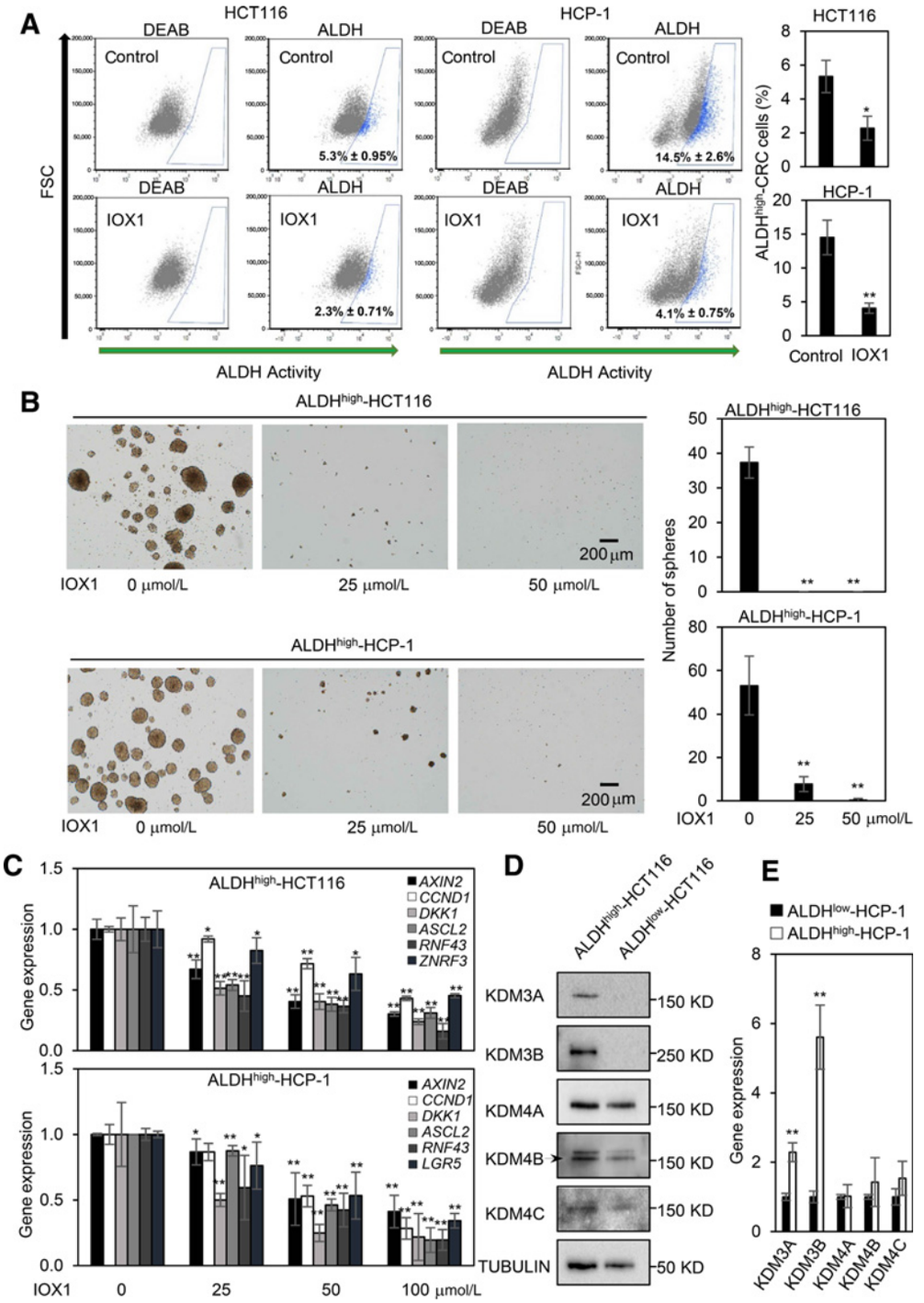

Figure 5.

IOX1 suppresses colorectal CSC-like properties through Wnt signaling. **A,** ALDEURF assay showed that ALDH^{high} CSC population was reduced in HCT116 or HCP-1 cells treated with 100 μmol/L of IOX1. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student *t* test ($n = 3$). **B,** Tumor sphere formation assay showed that the self-renewal ability was decreased by IOX1 in HCT116 and HCP-1 cells. Data represent mean ± SD of triplicate experiments. **, $P < 0.01$ by Student *t* test ($n = 3$). **C,** RT-qPCR showed that Wnt direct target gene expression was decreased in ALDH^{high}-HCT116 or ALDH^{high}-HCP-1 cells treated with IOX1. Data represent mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ by Student *t* test ($n = 3$). **D,** KDM3A and KDM3B were dramatically upregulated in ALDH^{high} CSCs of HCT116 compared with ALDH^{low} non-CSCs. **E,** KDM3A and KDM3B were significantly upregulated in ALDH^{high} CSCs of HCP-1 compared with ALDH^{low} non-CSCs. Data represent mean ± SD of triplicate experiments. **, $P < 0.01$ by Student *t* test ($n = 3$).

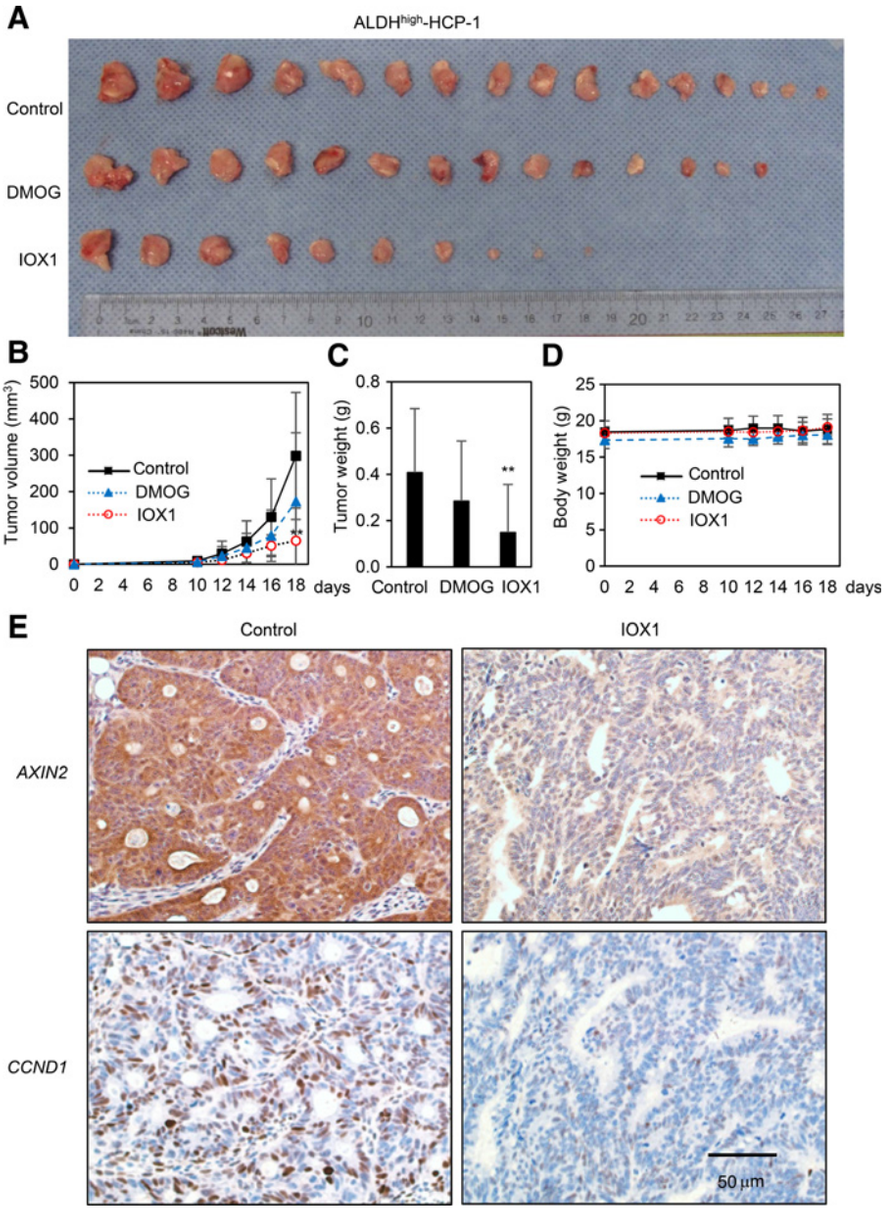

Figure 6.

IOX1 inhibits tumorigenesis of colorectal cancer *in vivo*. **A** and **B,** IOX1 significantly inhibited tumorigenic potentials of ALDH-^{high}-HCP-1 cells *in vivo*. **C,** Comparisons of tumor weights at the end of experiments. **D,** IOX1 had little effect on body weight change of the mice. **E,** Administration of IOX1 inhibited *AXIN2* and *CCND1* expression in HCP-1 xenografts. Immunostaining of HCP-1 xenografts using *AXIN2* and *CCND1* antibodies. Values are mean ± SD from a total of eight mice; **, $P < 0.01$, unpaired two-tailed Student *t* test ($n = 16$).

underline colon tumor initiation. Although *APC* and *CTNNB1* mutations play a decisive role in human colorectal cancer development, epigenetic alterations may co-promote colorectal cancer tumorigenesis with genetic mutations and are suggested to be biomarkers and therapeutic targets (40). Both KDM3 and KDM4 are reported to epigenetically control Wnt signaling through direct association with β-catenin on Wnt target gene promoters to modify H3K9 methylations, which family is more responsible for activation of Wnt target genes and related oncogenesis, especially in colorectal cancer, was not well characterized. The results from this study provide convincing evidence that suggest KDM4 is partially involved in regulating a subset of Wnt target expression in colorectal cancer, whereas KDM3 is more essential, indicating that KDM3 is more decisive than KDM4 in regulating oncogenic Wnt signaling in colorectal cancer. This may be partly because KDM3 can also help recruit co-activators of β-catenin to Wnt target gene promoters, such as MLL1 and BCL9 (11). In addition, KDM3B has been reported to interact with histone acetyltransferase CBP, which is also crucial in Wnt/β-catenin dependent transcriptional activation (7, 41, 42). Interestingly, both KDM3A and KDM3B showed elevated expression levels in ALDH^{high} CSCs as compared with ALDH^{low} non-CSCs, whereas KDM4 expression remained similar between CSCs and non-CSCs. CSCs are highly tumorigenic and are responsible for tumor propagation and relapse (43–46). This evidence further strengthened our finding that KDM3 may play a more essential role in oncogenic Wnt signaling in colorectal cancer.

Meanwhile, other studies have revealed that KDM4 family demethylases are also essential in the regulation of Wnt determined biological events. Of note, a recent study indicates that Wnt signaling can induce stabilization of KDM4C via inhibiting the ubiquitin proteasome pathway, and KDM4C can promote target gene expression and glioblastoma tumorigenesis through demethylation of H3K9,

which highlights that other regulatory mechanisms of these demethylases may also exist in modifying Wnt signaling in specific cancer types other than epigenetic regulation (13). In addition, KDM4A/B/C are also reported to be recruited by Brg1 to β-catenin/TCF4 transcription complex to promote liver regeneration in a mice model (34). Therefore, it is highly possible that the epigenetic control of Wnt target gene transcription mediated by these demethylases is more sophisticated than expected, and it can be context dependent, tissue-specific, or cancer-type related. However, in the case of Wnt-induced oncogenesis in colorectal cancer, although KDM3 and KDM4 are both involved, our study here demonstrates that KDM3 is more essential, implying that it may serve as an ideal therapeutic target in the eradication of colorectal cancer.

Wnt signaling has been demonstrated to play an important role in the initiation and development of colorectal cancer (1–4, 10). Numerous studies have already demonstrated and validated that the Wnt/β-catenin pathway is an attractive target for the treatment of colorectal cancer (9, 47). Notably, a milestone study has demonstrated that Apc restoration can trigger differentiation and restore intestinal hemostasis in established tumors with Kras and p53 mutations using a transgenic mouse model (9). The data indicate that Wnt activation is absolutely required to sustain tumor growth in advanced colorectal cancer and provide compelling evidence that Wnt signaling may be a promising therapeutic target for treatment of colorectal cancer (9). However, no FDA-approved drug is available in the clinic for treatment that targets the Wnt signaling pathway, despite substantial effort invested into therapeutic development of Wnt inhibitors in the past two decades (4, 47–49). Our studies revealed that IOX1 can significantly suppress Wnt target gene transcription and Wnt-induced tumorigenesis in colorectal cancer. The unbiased RNA-seq and GSEA indicated that IOX1 treatment leads to global inhibition of Wnt signature gene expression in colorectal cancer cells with *APC* or *CTNNB1* mutations, which are the major causes of the initiation of human colorectal cancer. IOX1 has been suggested as a broad-spectrum inhibitor of JmjC domain-containing histone demethylases and it has strong inhibitory effect against KDM3, KDM4, and KDM6 (18, 19). On the basis of our previous studies, it is unlikely that KDM6 is involved in Wnt signaling (11). Therefore, IOX1 should suppress Wnt signaling through the inhibition of KDM3 and KDM4. In agreement with this, our ChIP data strongly suggest that IOX1 suppresses Wnt target gene transcription primarily through inhibition of H3K9me2 demethylation that is catalyzed by KDM3 and/or KDM4. In addition, the molecular-modeling studies further support this conclusion by showing that mechanistically IOX1 can bind to the JmjC domain and thereby inhibit the enzymatic activity of these histone demethylases. It has been reported that IOX1 represents the strongest inhibitory activity against KDM3A based on *in vitro* measurement of the histone demethylase activities (18). Therefore, it is highly possible that it may also present a similar behavior in colorectal cancer cells. By using RT-PCR, we demonstrated that IOX1 can still dramatically suppress Wnt direct key oncogene targets expression without KDM4 instead of KDM3 (**Fig. 3E**). These evidences strongly suggest that IOX1 suppresses colorectal cancer tumorigenesis primarily through inhibition of KDM3 demethylases.

CSCs, defined by their capacity of self-renewal and resistance to conventional chemotherapies, are responsible for tumor propagation and relapse (43). Targeting CSCs represents a new strategy in cancer treatment. IOX1 has the potential to suppress the CSC-like properties both *in vitro* and *in vivo*, which highlights a novel therapeutic approach to eliminate CSCs in colorectal cancer for a more efficient strategy of cancer therapy. Indeed, based on our *in vitro* assays, IOX1 had strong inhibitory effects in suppressing colorectal cancer oncogenic growth and stemness in the range of 25–50 μmol/L. This concentration range is consistent with other studies using IOX1 to treat different types of cancer cell lines (19, 20). Although its broad-spectrum nature may prevent it from being developed into a drug, these limitations may be overcome by modifying its chemical structure to generate novel derivatives that are more potent and selective. Therefore, we should not exclude IOX1 as a potential lead for future drug design and discovery. Our computational chemistry studies may provide further insight into design of more selective small-molecule inhibitors based on the IOX1 skeleton.

In conclusion, we have discovered that KDM3 family histone demethylases may exert a more essential role than KDM4 in the epigenetic control of the Wnt/β-catenin–dependent transcriptional activation in colorectal cancer by characterization of the functional redundancy and differences between KDM3 and KDM4. In addition, we also discovered that the small-molecule inhibitor, IOX1, potently suppresses Wnt target gene transcription and Wnt-induced tumorigenesis in colorectal cancer primarily through inhibiting the enzymatic activity of KDM3. IOX1 has the potential as a lead to help develop a novel therapeutic approach for the eradication of colorectal cancer, which is still a life-threatening disease without an effective targeted therapeutic strategy at present. Collectively, our findings not only provide new insights into the epigenetic regulation of Wnt target gene transcription but also a novel and potential therapeutic approach for eradication of colorectal cancer.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

R.G. Hoyle: Data curation, validation, investigation, visualization, writing-original draft, writing-review and editing. **H. Wang:** Software, validation, investigation, methodology, writing-original draft, writing-review and editing. **Y. Cen:** Writing-review and editing. **Y. Zhang:** Software, supervision, funding acquisition, validation, methodology, writing-original draft, writing-review and editing. **J. Li:** Conceptualization, data curation, supervision, funding acquisition, validation, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing.

Acknowledgments

This work was partially supported by NIH/NIDCR grant (R03DE026822), Elsa U. Pardee Foundation Award, 2020 VCU Presidential Research Quest Fund, VCU Massey Cancer Center Multi-Investigator Award (2019-MIP-05), 2020 VCU CCTR Endowment Fund (sub-award of the UL1TR002649 from National Center for Advancing Translational Sciences to VCU's CTSA; to J. Li), and NIH/NIDA R01DA024022 and R01DA044855 (to Y. Zhang). Services in support of the research project were provided by the VCU Massey Cancer Center Flow Cytometry Core, supported, in part, with funding from NIH-NCI Cancer Center support grant P30 CA016059.

Received April 26, 2020; revised September 2, 2020; accepted October 21, 2020; published first November 17, 2020.

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