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ACTL6A-mediated histone modifications regulate global gene expression in gastrointestinal cancer

위장관암에서 히스톤 변형을 통해 유전자 발현을 조절하는 ACTL6A의 기전 규명

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2021년 1월

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ACTL6A-mediated histone modifications regulate global gene expression in gastrointestinal cancer

by

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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science

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Abstract

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The mammalian SWI/SNF (Switching defective/sucrose non-fermentable) chromatin remodeling complex is mutated in over 20% of human cancer [1]. Mechanistic studies on various SWI/SNF complex subunits have been conducted to further understand SWI/SNF complex-mediated chromatin regulatory process. Better understanding of each SWI/SNF complex subunit's epigenetic role in cancer could present novel and subunit-specific therapeutic opportunities. Actin-like protein 6A (ACTL6A), also known as BAF53a, is a subunit of SWI/SNF chromatin complex. ACTL6A was previously identified as an essential epigenetic modifier via CRISPR/Cas9 knockout library screening in gastrointestinal cancer [2]. However, the underlying mechanisms of ACTL6A in gastrointestinal cancer remain unknown. In the present study, I aim to identify the molecular mechanisms of epigenetic modifier ACTL6A in relation to SWI/SNF chromatin remodeling complex.

Depletion of ACTL6A by CRISPR/Cas9 system and shRNA inhibition significantly impedes cell growth in gastrointestinal cancer. Introduction of CRISPR/Cas9-resistant ACTL6A clone in reversibility assay recovers cell growth. ACTL6A inhibition in mouse xenograft model i*n-vivo* also shows decreased tumor growth, further verifying the cell proliferation regulating role of ACTL6A.

Functional study of ACTL6A in relation to SWI/SNF complex reveals that the depletion of ACTL6A is more effective in cell growth inhibition compared to other SWI/SNF complex subunits such as SMARCA4, SMARCA2, and ARID1A. As a subunit of SWI/SNF complex, ACTL6A regulates protein stability of the catalytic subunits SMARCA4/2. Expression

and nuclear localization of ACTL6A is consistent in SMARCA4/2 proficient and SMARCA4/2 deficient cell lines. I demonstrate that ACTL6A is not part of the SWI/SNF subcomplex that lacks SMARCA4/2, which is consistent with the findings from previous studies [3,4]. Interestingly, loss of function study of ACTL6A in SMARCA4/2 proficient and SMARCA4/2 deficient cell lines shows that ACTL6A inhibition effectively arrests cell proliferation regardless of SMARCA4/2 presence. This results suggests an additional role of ACTL6A, independent from SWI/SNF complex.

Transcriptome sequencing of ACTL6A knockout cells indicates that ACTL6A regulates genome wide transcription via chromatin remodeling. ChIP-seq of ACTL6A knockout cells showed decreased genome-wide enrichment of histone activation marker, H3K27Ac. Collectively, my study presents that ACTL6A regulates genome-wide histone modifications and global gene transcription in gastrointestinal cancer.

Key Words: ACTL6A/BAF53a/Arp4; SWI/SNF complex; SMARCA4/Brg1; SMARCA2/BRM; Histone modification; Transcription regulation; Epigenetics; Gastrointestinal cancer;

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Introduction

Epigenetic regulations that induce change in gene expression without permanent modifications in the DNA sequence are commonly linked to cancer pathogenesis [5,6]. Advances in the field of chromatin structure have made identification and mapping of epigenetic alteration such as DNA methylation, covalent histone modification, and chromatin remodeling possible [6-8]. Research in cancer epigenomics is providing novel therapeutic strategies, due to the reversible and dynamic nature of epigenetic modification [5,6].

Abnormal expression and epigenetic regulation of chromatin remodeling complex in cancer cells can initiate and maintain oncogenic properties [7]. SWI/SNF complex is one of the chromatin remodeling complexes that depends on ATP hydrolysis to move, exchange, or remove nucleosome to regulate chromatin architecture [7,9]. SWI/SNF complex is composed with various subunits, including the functional ATPase subunit SMARCA4 and SMARCA2 [10]. SWI/SNF complex is known as the most frequently mutated chromatin regulator, with over 20% mutation in human cancer [1,11]. Mechanistic studies on various SWI/SNF subunits have been conducted to further understand the role of different subunits in genome-wide chromatin organization and to identify subunit-specific synthetic lethalities [12].

Actin-like protein 6A (ACTL6A), also known as BAF53a, is a subunit of SWI/SNF chromatin complex. Previous study identified ACTL6A as an essential epigenetic modifier via CRISPR/Cas9 knockout screening in gastrointestinal cancer [2]. ACTL6A is known to be upregulated in various cancers such as colon cancer, glioma, and osteocarcinoma and has been indicated as a marker of poor prognosis in cancer patients [13-16]. Previous studies show that ACTL6A enhance cancer cell survival, and suggest a role of ACTL6A in various cellular process including epithelial-mesenchymal transition, metastasis, cyclin dependent kinase inhibition, and c-Myc interacting nuclear complex formation [13-17]. However, the epigenetic and molecular mechanism of ACTL6A in gastrointestinal cancer remains unknown.

In this functional study of the identified target ACTL6A, I investigate the molecular mechanism of epigenetic modifier ACTL6A in relation to SWI/SNF chromatin complex. While the chromatin remodeling mechanisms of SWI/SNF complex have been analyzed, specific role of ACTL6A within SWI/SNF complex has not yet been defined, especially in gastrointestinal cancer [9]. The function of SWI/SNF complex depends on the ATPase activity of SMARCA4/2. While ACTL6A lacks ATPase function, I show that the loss of ACTL6A is more effective in cell growth inhibition than SMARCA4/2. I report that ACTL6A is a critical epigenetic modifier and SWI/SNF subunit, with SWI/SNF complex independent mechanism of regulating gastrointestinal cancer cell proliferation via genome-wide histone modification and chromatin regulation.

Materials and Methods

Cell culture & reagents

Cells were obtained from American Tissue Culture Collection or the Korean Cell Line Bank. Cells were cultured in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum and gentamicin (10μg/mL) at 37°C in a 5% CO_2-humidified atmosphere. Cells were regularly tested for mycoplasma contamination. Cells were treated with MG-132 (Sigma-Aldrich, #M7449) at 5 or 10uM for 8h for proteasome inhibition. For FITC Annexin V staining, cell suspensions were incubated with Annexin V and propidium iodide according to the manufacturer's protocol (BD Pharmingen, #556547).

Constructs

pBABE-puro (Addgene, 1764) and MSCV-PIG (Addgene, 18751) backbones were used for overexpression cloning. TRC lentiviral shRNAs for ACTL6A (Horizon, TRCN0000072273, TRCN0000072274) were used for shRNA inhibition, and to create doxycycline-inducible clone in Tet-pLKO-puro plasmid (Addgene, 21915). Wild-type ACTL6A sequence was subcloned from pBS-hBAF53a (Addgene, 17879). CRISPR-resistant ACTL6A clone was created using the site-directed mutagenesis kit QuickChange-II (Agilent Technologies, #200523). Primer sequences used to create CRISPR-resistant ACTL6A clone are noted in Table 1. GFP-SMARCA4 plasmid (Addgene, 65391) was used for SMARCA4 overepxression.

Table 1. Primer sequence for site-directed mutagenesis of ACTL6A

Gene	(Sequence 5' → 3')	
CRISPR-resistant ACTL6A #4	F	caatgccttgttgaaggacgtatccatcgtggactggaattg
	R	caattccagtccacgatggatacgtccttcaacaaggcattg

Virus production and transduction

Two distinct single guide RNAs targeting each gene or control GFP were cloned into LentiCRISPRv2 vector system (Addgene, 52961). Viral vectors were transfected into 293FT cells using Virapower packaging mix (Invitrogen) as described previously [18]. The viruses were harvested after 48 hours and transduced to target cells in the presence of 6 μg/ml polybrene (Sigma-Aldrich, #H9268). After 24 hours of incubation, the transduced cells were selected in 1 μg/ml puromycin (Sigma-Aldrich, #P8833) for 7 days. Knockdown of target genes was validated by western blot analysis. To make single clones, transduced cells were plated into 96-well plates. After approximately 30 days of clonal expansion, silencing or overexpression was confirmed by immunoblotting. The oligo sequences used for sgRNA are noted in Table 2.

Table 2. Primer sequence for sgRNA

Gene	(Sequence 5' → 3')	
ACTL6A-4	F	caccgGTTGAAGGACATAGCCATCG
	R	aaccCGATGGCTATGTCCTTCAACc
ACTL6A-5	F	caccgTGCCAAGACCTCGTAACCTG
	R	aaccCAGGTTACGAGGTCTTGGCAc
SMARCA4-1	F	caccgCTGGCCGAGGAGTTCCGCCC
	R	aaccGACCGGCTCCTCAAGGCGGGc
SMARCA4-3	F	caccgCCTGTTGCGGACACCGAGGG
	R	aaccGGACAACGCCTGTGGCTCCCc
SMARCA2-49	F	caccgTCGCATACCAGTGCCCTTCA
	R	aaccTGAAGGGCACTGGTATGCGAc
SMARCA2-50	F	caccgAAGTCTGTGGACCCCATCGT
	R	aaccACGATGGGGTCCACAGACTTc
SMARCA2-92	F	caccgCTTGTCATGTATACCATCGA
	R	aaccTCGATGGTATACATGACAAGc
ARID1A-1	F	caccgGCGGTACCCGATGACCATGC
	R	aaccGCATGGTCATCGGGTACCGCc
ARID1A-2	F	caccgATGGTCATCGGGTACCGCTG
	R	aaccCAGCGGTACCCGATGACCATc

Western blot analysis

Cultured cells were washed with phosphate buffered saline (PBS) and lysed with lysis buffer (50 mM Tris-HCl (pH 7.5), 1 % NP-40, 0.1 % sodium deoxycholate, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM EDTA and protease/phosphatase inhibitors). Lysates were collected after centrifugation at 13,000 rpm for 20 min. Protein concentrations were quantified with a Bicinchoninic Acid Protein Assay Reagent (Pierce, #23225), according to manufacturer's instructions. Western blot analysis was performed as previously described [19]. Antibodies to the following were used: ACTL6A (Bethyl, #A301-391A); Caspase 3 (Cell Signaling Technologies, #9662); Cleaved-Caspase 9 (Cell Signaling Technologies, #7237); Actin (Santa Cruz, #sc1616); HA (Abcam, #9110); SMARCA4 (Abcam, #110641; Santa Cruz, #sc17796); SMARCA2 (Cell Signaling Technologies, #11966); SMARCB1 (Bethyl, #A301-087A); GFP (Santa Cruz, #sc9996); ARID1A (Bethyl, #A301-041A); SMARCC1 (Santa Cruz, #sc32763); SS18 (Cell Signaling Technologies, #21792).

Colony formation assay

Colony formation assays were performed in 6-well plates. Cells were plated in triplicate at a density of 6×10^3 or 1.5×10^4 cells/well. Seven to ten days later, the medium was removed and the cells were fixed and stained with Coomassie Brilliant Blue solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid).

Animal studies

All animal studies were performed in accordance with protocols approved by the Seoul National University Institutional Animal Care and Use Committee (IACUC). Xenograft tumors were generated in 4-week-old female balb/c nude mice (OrientBio) by subcutaneous injection of 1.0 x 10^7 AGS or 5.0 x 10^6 HCT116 cells suspended in 200ul 50% matrigel (BD Bioscience, #356234). For the doxycycline-treated tumor group, mice were given 1mg/ml Doxycycline (Sigma-Aldrich, #D9891) and 5% sucrose in drinking water for three months. Tumor volumes were calculated using the formula: tumor volume (mm^3) = 1/2 ($length^2$ x width).

RNA extraction and RNA-seq

Total RNA was extracted using TRI reagent (Molecular Research Center, #TR-118) in accordance with the manufacturer's instructions. Total RNA (2μg) was reverse transcribed for cDNA synthesis and real-time qPCR analysis were performed as previously described [19]. Primer sequences used for RT-qPCR are noted in Table 3. Sequencing libraries were generated according to the standard protocol of Illumina Inc. for high-throughput sequencing. The transcriptome was then sequenced using a Genome Analyzer IIx (Illumina Inc.) as previously described [20]. Sequenced reads were aligned to human transcript reference sequences from the UCSC database (Homo_sapiens.GRCh37/hg19) for expression analysis. Differentially expressed genes (DEGs) were identified according to overall differential expression from the DEGseq analysis with FDR <0.001.

Table 3. Primer sequence for quantitative Real-Time PCR

Gene	(Sequence 5' → 3')	
ACTL6A	F	CAGAGGCACCGTGGAATACT
	R	AGGACATAGCCATCGTGGAC

Immunoprecipitation

Nuclear extract was obtained by incubating trypsinized cells first in Buffer A (10mM Tris-HCl (pH7.5), 10mM NaCl, 3mM MgCl2, and protease/phosphatase inhibitors) for 15 minutes, followed by addition of 10% NP40. The pellets were collected after centrifugation at 2000 rpm for five minutes, and resuspended in Buffer B (10mM Tris-HCl (pH 7.5), 10mM KCl, 1mM DTT, 1mM MgCl2, and protease/phosphatase inhibitors). The pellets were collected and lysed with Buffer C (50mM Tris-HCl (pH7.5), 150mM NaCl, 1mM sodium pyrophosphate, 1% NP-40, 0.1% sodium deoxycholate, and protease/phosphatase inhibitors). Total nuclear extract was pre-cleared with Dynabead (Invitrogen, #10004D, #10002D) for four hours, and incubated overnight in 4°C with Dynabead and 5~10ug antibodies or IgG. Antibodies to the following were used: Rabbit IgG (Abcam, ab37415); Rabbit Anti-HA tag (Abcam, ab9110); Mouse IgG (Abcam, ab37355); Mouse Anti-HA tag (Abcam, ab18181); SMARCB1 (Bethyl, A301-087A).

Chromatin immunoprecipitation (ChIP) assay and ChIP-seq

ChIP assays were determined as previously described [19]. For CHIP-seq, reads were mapped to the reference genome by bowtie2 ver 2.3.2 software [21]. MACS2 ver 2.1 was used to identify significantly enriched regions of point source factor or broad source factor. Annotation and functional analysis step were performed by ChIPpeakAnno ver 3.12.0 and ChIP-Enrich ver 2.2.0 packages in R [22,23]

Bioinformatics analyses

RNA-seq data of three differentially expressed gene cluster sets was used to conduct gene set enrichment analysis according to the GSEA user manual (http://software.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html). GSEA was then performed against Gene Ontology gene sets using the default parameters.

Results

Loss of ACTL6A inhibits cell proliferation and induces apoptosis in gastrointestinal cancer.

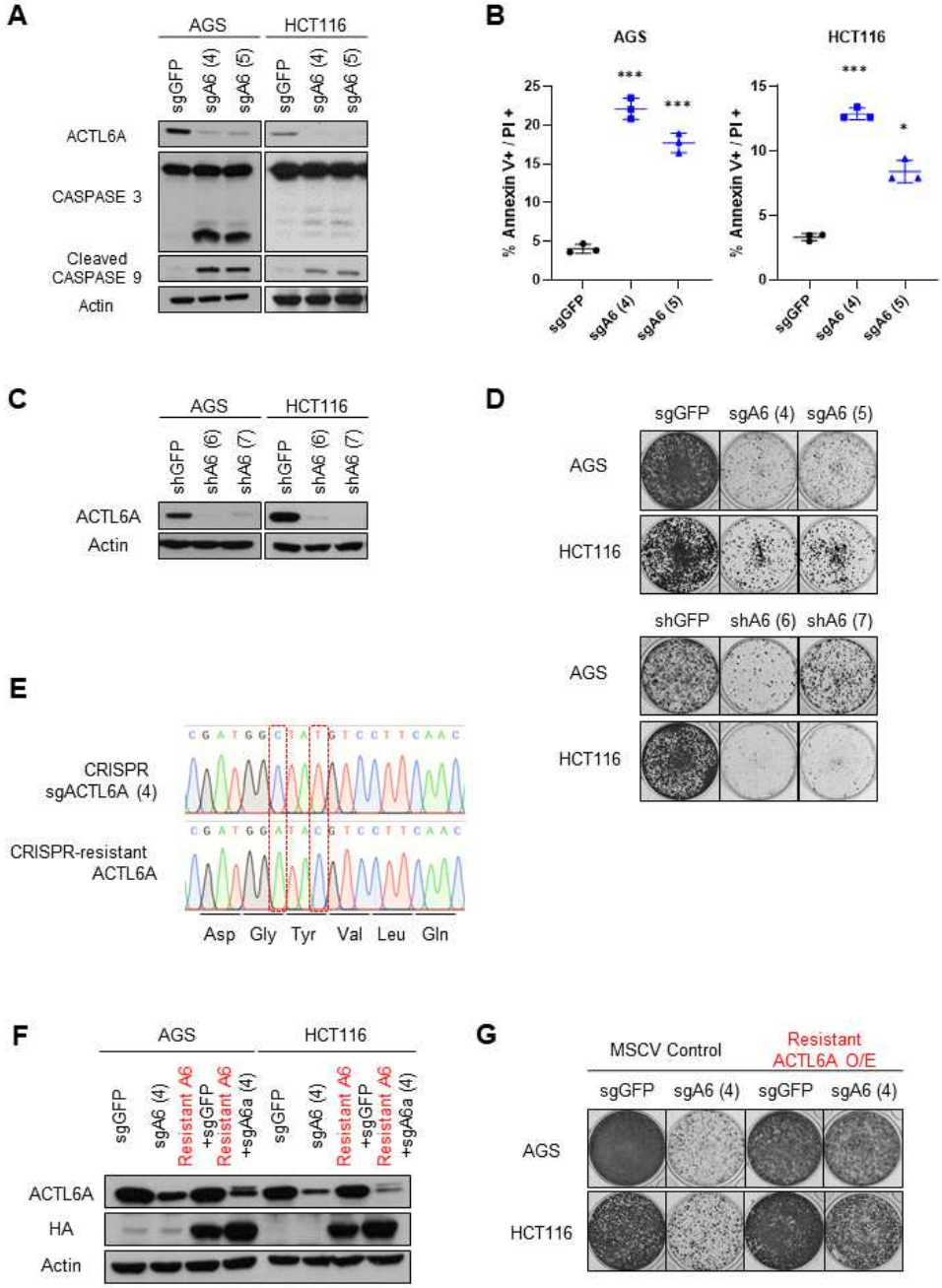
Previously, ACTL6A was identified as an essential epigenetic modifier in gastric and colon cancer, since knockout of ACTL6A by dual sgRNA-directed CRISPR/Cas9 effectively inhibited cell proliferation [2]. To confirm the specificity of ACTL6A knockout, ACTL6A was depleted using single sgRNA-directed CRISPR/Cas9 for two different ACTL6A sgRNAs. Loss of ACTL6A by both single sgRNAs caused significant proliferative arrest in AGS and HCT116 cell lines (Figures 1A and 1D). Compared to the control cells, ACTL6A depleted cells had higher percentage of annexin V and propidium iodide positive cells, which is indicative of apoptosis (Figure 1B). Western blot of ACTL6A depleted cells showed upregulated cleaved caspase 3 and 9 expression, which also suggests that the loss of ACTL6A induces apoptosis in gastrointestinal cancer (Figure 1A).

Next, on-target effects of ACTL6A loss was further validated, using shRNA inhibition. shRNA-mediated ACTL6A depletion also effectively inhibited cell growth in AGS and HCT116 (Figures 1C and 1D). CRISPR/Cas9-resistant ACTL6A clone was created by site-directed mutagenesis of two nucleotides, without alteration of amino acid sequence (Figure 1E). Introduction of CRISPR/Cas9 resistant ACTL6A clone overcame the cell proliferation inhibiting effects of CRISPR/Cas9 induced ACTL6A knockout (Figures 1F

and 1G). Collectively, my results confirmed the on-target effects of CRISPR/Cas9 induced ACTL6A depletion.

Next, I examined the effects of ACTL6A inhibition on tumor growth using mouse xenograft model *in vivo*. AGS cell lines with doxycycline-inducible shRNAs targeting ACTL6A was initially tested *in vitro*. Upon doxycycline treatment, ACTL6A mRNA expression and cell proliferation decreased (Figures 2A and 2B). Confirmed AGS cell lines with inducible shRNAs targeting either ACTL6A or control GFP were subcutaneously injected into balb/c nude mice for tumor formation. Doxycycline was given via drinking water to the treatment group and tumor growth was monitored for 6 weeks (Figure 2C). Doxycycline-activated ACTL6A depletion reduced tumor growth in tumor xenografts (Figures 2D and 2E). Protein samples obtained from the mouse tumor after the completion of *in vivo* study confirmed that ACTL6A was depleted in doxycycline-treated mouse (Figure 2F). Thus, ACTL6A is required for tumor proliferation *in vivo*, which further validates the cell proliferation regulating role of ACTL6A.

Fig.1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AGS
HCT116
sgGFP
sgA6 (4)
sgA6 (5)
ACTL6A
CASPASE 3
Cleaved CASPASE 9
Actin
B
AGS
HCT116
% Annexin V+ / PI +
***
*
sgGFP
sgA6 (4)
sgA6 (5)
C
AGS
HCT116
shGFP
shA6 (6)
shA6 (7)
ACTL6A
Actin
D
sgGFP
sgA6 (4)
sgA6 (5)
AGS
HCT116
shGFP
shA6 (6)
shA6 (7)
E
CRISPR sgACTL6A (4)
CRISPR-resistant ACTL6A
Asp Gly Tyr Val Leu Gln
F
AGS
HCT116
sgGFP
sgA6 (4)
Resistant A6 +sgGFP
Resistant A6 +sgA6a (4)
ACTL6A
HA
Actin
G
MSCV Control
Resistant ACTL6A O/E
sgGFP
sgA6 (4)
AGS
HCT116

Figure 1. Effects of ACTL6A depletion in gastrointestinal cell proliferation.

(A) Western blot analysis of AGS and HCT116 cells transduced with indicated sgRNAs, where sgA6 represent ACTL6A sgRNA and sgGFP represent GFP sgRNA used as negative control. Whole cell extracts were prepared on day 7 post transduction. Actin served as a loading control.

(B) FITC-Annexin V staining after the depletion of ACTL6A to quantify apoptosis. Percentages of apoptotic cells with positive Annexin V and propidium iodide (PI) staining are shown with horizontal bar indicating mean and error bars indicating ±SD (n=3). *p ≤ 0.05, ***p ≤ 0.001, unpaired t test.

(C) Western blot analysis of AGS and HCT116 cells transduced with indicated shRNAs. Whole cell extracts were prepared on day 7 post transduction.

(D) Colony formation assay after ACTL6A depletion.

(E) CRISPR/Cas9-resistant mutant ACTL6A clone has two point mutations. Sanger sequencing results of sgRNA ACTL6A #4 target site in wild type and CRISPR/Cas9-resistant clone is shown.

(F) Western blot analysis of reversibility test using CRISPR/Cas9-resistant clone. Single-cell clones with CRISPR/Cas9-resistant ACTL6A was screened in puromycin, followed by transduction of ACTLA sgRNA and blasticidin selection.

(G) Colony formation assay after ACTL6A depletion in MSCV control cell and CRISPR/Cas9-resistant ACTL6A single-cell clone.

Fig.2

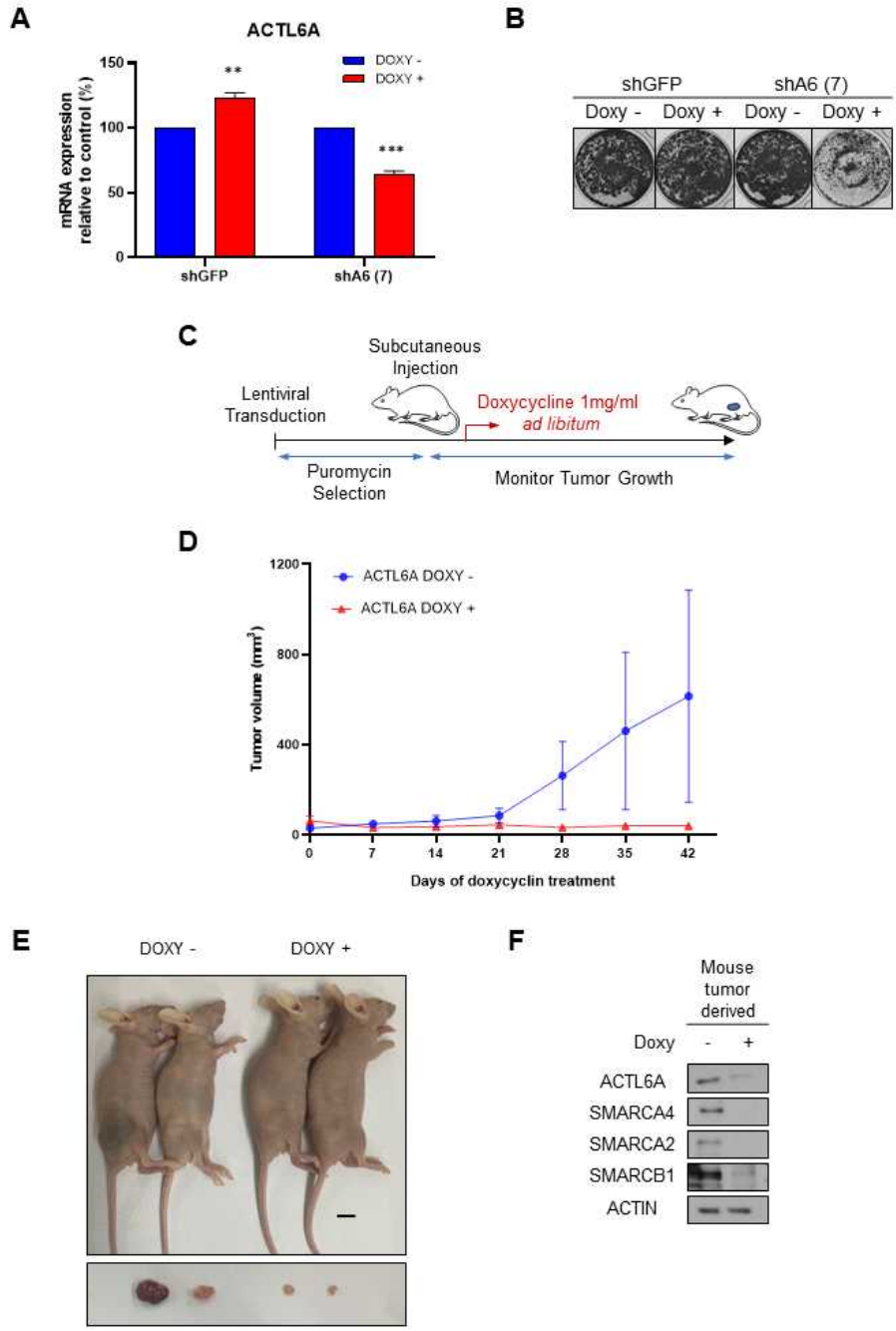
A
ACTL6A
DOXY -
DOXY +
mRNA expression relative to control (%)
150
100
50
0
**
***
shGFP
shA6 (7)
B
shGFP
shA6 (7)
Doxy -
Doxy +
Doxy -
Doxy +
C
Subcutaneous Injection
Lentiviral Transduction
Doxycycline 1mg/ml
ad libitum
Puromycin Selection
Monitor Tumor Growth
D
ACTL6A DOXY -
ACTL6A DOXY +
Tumor volume (mm³)
1200
800
400
0
0
7
14
21
28
35
42
Days of doxycyclin treatment
E
DOXY -
DOXY +
F
Mouse tumor derived
Doxy
-
+
ACTL6A
SMARCA4
SMARCA2
SMARCB1
ACTIN

Figure 2. ACTL6A is required for cell proliferation *in vivo*.

(A) Effects of doxycycline (doxy) treatment in AGS cell lines harboring doxycycline-inducible shRNAs targeting GFP or ACTL6A. Doxycycline (1ug/ml) was treated for three days before RNA extraction. mRNA levels were normalized to 18s level. qRT-PCR analysis results shown with horizontal bar indicating mean and error bars indicating SD (n=3). **p ≤ 0.01, ***p ≤ 0.001, unpaired t test.

(B) Colony formation assay after doxycycline treatment.

(C) Schematic diagram of the *in vivo* experiment. AGS cells were injected subcutaneously into balb/c nude mice. Where indicated, mice were given 1mg/ml doxycycline in drinking water for the duration of the experiment.

(D) Tumor volume was measured with calipers at the indicated time points after doxycycline treatment. Error bars corresponds to mean ± s.e.m (n= 4).

(E) Representative images of balb/c nude mouse and tumor measured 42 days after doxycycline treatment. Scale bars, 10mm.

(F) Western blot analysis of mouse tumor derived protein. Actin served as a loading control.

SWI/SNF complex subunit ACTL6A regulates SMARCA4/2 protein stability.

ACTL6A is a known subunit of SWI/SNF complex and has been reported to be commonly amplified in multiple cancers, which suggests that ACTL6A may act as an oncogenic driver via SWI/SNF complex [24]. Co-immunoprecipitation of HEK293 cell with HA-tagged ACTL6A and GFP-tagged SMARCA4, as well as co-immunoprecipitation of AGS cell with HA-tagged ACTL6A confirmed interaction between ACTL6A and SWI/SNF complex subunits (Figures 3A and 3B).

Chromatin remodeling activity of SWI/SNF complex is ATP dependent, and therefore requires the functional subunits SMARCA4/2 to catalyze ATP hydrolysis [10]. I tested whether ACTL6A depletion effects SMARCA4/2 expression, which would consequently modify the chromatin remodeling activity of SWI/SNF complex. ACTL6A depleted cells showed decreased SMARCA4 and/or SMARCA2 protein expression, which was also seen in colon cancer organoid and mouse tumor derived cells (Figures 3C, 3D and 2F). This results were consistent with the previous study that showed ACTL6A depletion induced SMARCA4 degradation [25]. Treatment of proteasome inhibitor MG132 after ACTL6A knockout partially reversed the protein degradation of SMARCA4 (Figure 3F). However, transcriptome sequencing revealed that knockout of ACTL6A had no significant change in RNA expression of SWI/SNF complex subunits including SMARCA4/2 (Figure 3G). Taken together, these results indicate that ACTL6A is a critical SWI/SNF complex subunit that regulates protein stability of SMARCA4/2.

Fig.3

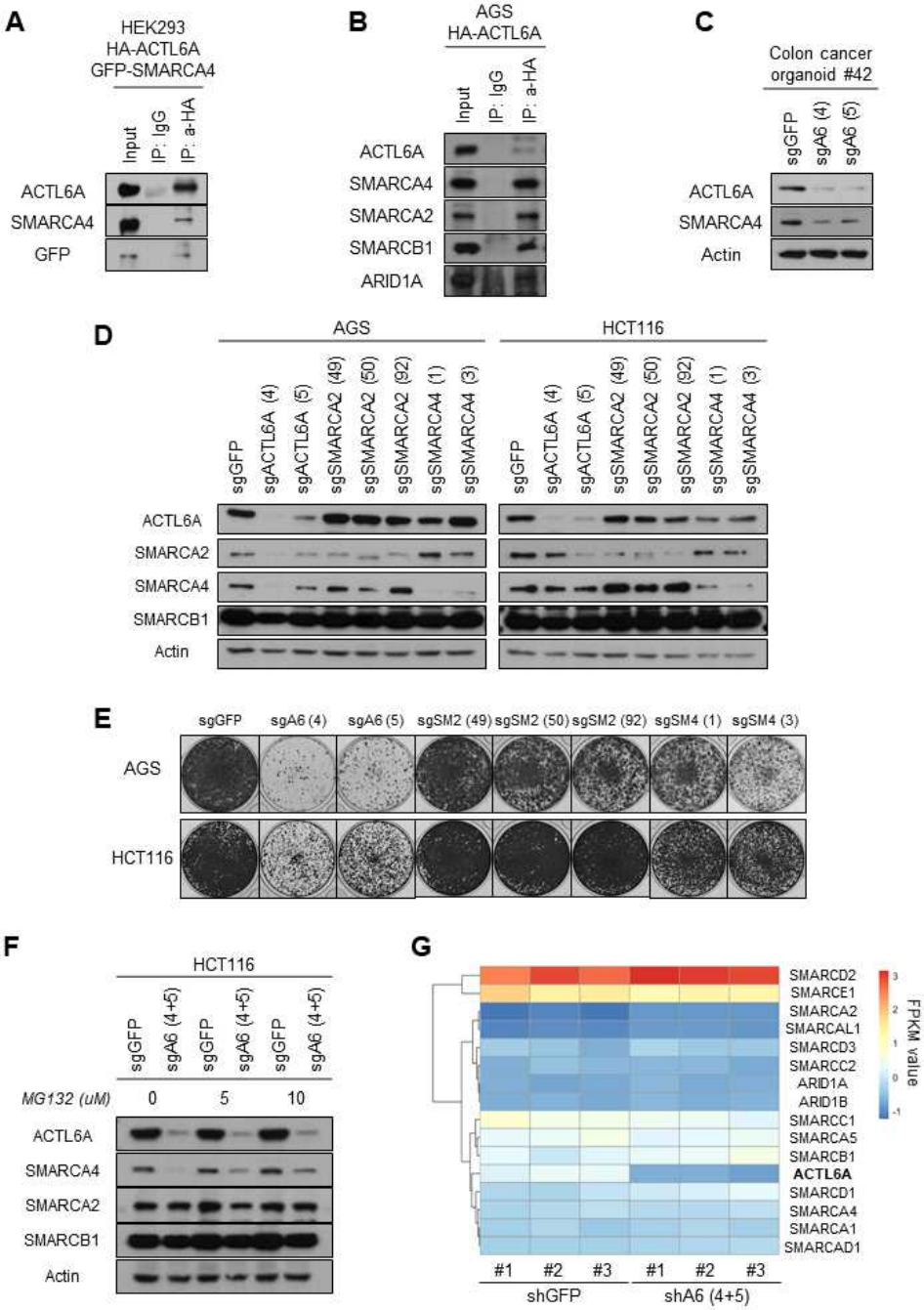
A
HEK293
HA-ACTL6A
GFP-SMARCA4
Input
IP: IgG
IP: a-HA
ACTL6A
SMARCA4
GFP
B
AGS
HA-ACTL6A
Input
IP: IgG
IP: a-HA
ACTL6A
SMARCA4
SMARCA2
SMARCB1
ARID1A
C
Colon cancer organoid #42
sgGFP
sgA6 (4)
sgA6 (5)
ACTL6A
SMARCA4
Actin
D
AGS
HCT116
sgGFP
sgACTL6A (4)
sgACTL6A (5)
sgSMARCA2 (49)
sgSMARCA2 (50)
sgSMARCA2 (92)
sgSMARCA4 (1)
sgSMARCA4 (3)
ACTL6A
SMARCA2
SMARCA4
SMARCB1
Actin
E
sgGFP
sgA6 (4)
sgA6 (5)
sgSM2 (49)
sgSM2 (50)
sgSM2 (92)
sgSM4 (1)
sgSM4 (3)
AGS
HCT116
F
HCT116
sgGFP
sgA6 (4+5)
MG132 (uM)
0
5
10
ACTL6A
SMARCA4
SMARCA2
SMARCB1
Actin
G
SMARCD2
SMARCE1
SMARCA2
SMARCAL1
SMARCD3
SMARCC2
ARID1A
ARID1B
SMARCC1
SMARCA5
SMARCB1
ACTL6A
SMARCD1
SMARCA4
SMARCA1
SMARCAD1
FPKM value
#1
#2
#3
shGFP
shA6 (4+5)

Figure 3. SWI/SNF complex subunit ACTL6A regulates SMARCA4/2 protein stability.

(A) Co-immunoprecipitation using the nuclear extract of HEK293 cell transfcted with HA tagged ACTL6A and GFP tagged SMARCA4.

(B) Co-immunoprecipitation using the nuclear extract of AGS single-cell clone with HA tagged ACTL6A.

(C) Western blot analysis of colon cancer organoid (#42) transduced with indicated sgRNAs.

(D) Western blot analysis of AGS and HCT116 cells transduced with indicated sgRNAs. Whole cell extracts were prepared on day 7 (AGS) or day 9 (HCT116) post transduction. Actin served as a loading control.

(E) Colony formation assay after depletion of indicated sgRNAs.

(F) Western blot analysis after MG132-induced proteosomal inhibition in ACTL6A depleted HCT116 cell.

(F) Transcriptome sequencing results of ACTL6A depleted HCT116 cell. RNA expression of SWI/SNF complex subunits are shown in FPKM values.

ACTL6A is a critical SWI/SNF complex subunit that regulates genome-wide gene transcription.

Effects of ACTL6A inhibition on functional subunits of SWI/SNF suggest that ACTL6A could cause abnormal cancer cell proliferation via SMARCA4/2 modification. Therefore, I compared the effects of ACTL6A, SMARCA4, and SMARCA2 depletion in AGS and HCT116. While previous studies on SMARCA4/2 state that depletion of SMARCA4/2 results in cell cycle arrest, my results showed that the proliferation inhibiting effects of ACTL6A was more effective and evident in earlier time point than SMARCA4/2 (Figure 3E). Whole transcriptome sequencing showed that the inhibition of ACTL6A resulted in 1115 differentially expressed genes (DEGs), while the inhibition of SMARCA4 or 2 resulted in significantly less DEGs (Figures 4A, 4B and 4C). It should be noted that individual depletion of either SMARCA4 or SMARCA2 may not present significant effects on SMARCA4/2 proficient cell lines such as HCT116 since two subunits are mutually exclusive. Loss of SMARCA4 has been found to increase dependency on SMARCA2, suggesting how two can compensate for each other [3, 26]. Regardless, ACTL6A has a more critical and essential role in gastrointestinal cell growth compared to SMARCA4/2.

GEO analysis of the DEGs of ACTL6A knockout cells showed enrichment in chromosome or chromatin organization, which corresponds with SWI/SNF complex function (Figure 4D). Interestingly, less than 3% of the ACTL6A DEGs overlapped with SMARCA4/2 DEGs (Figure 4C). This suggests the existence of both SWI/SNF complex dependent and independent functions of ACTL6A.

Fig.4

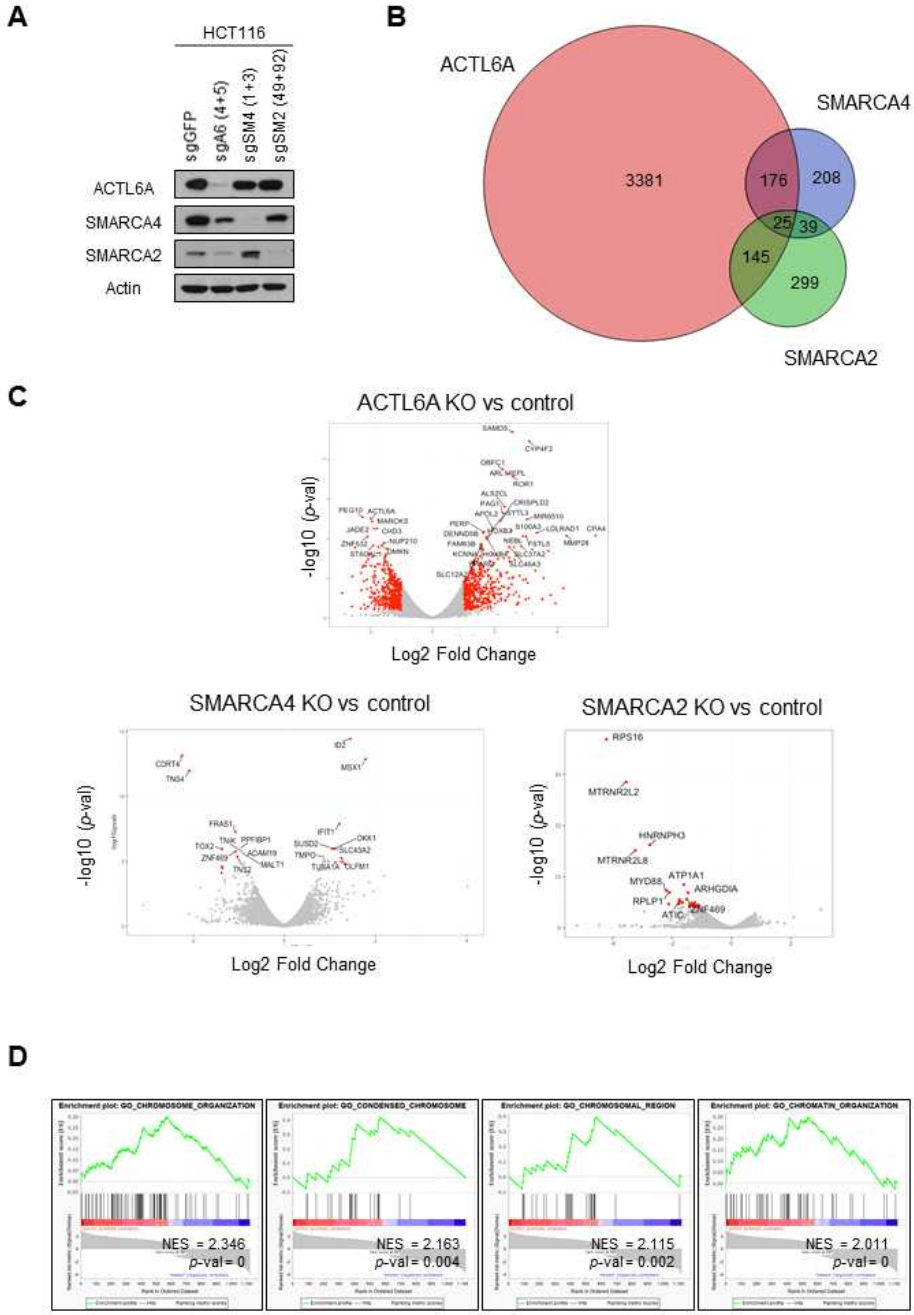
A
HCT116
sgGFP
sgA6 (4+5)
sgSM4 (1+3)
sgSM2 (49+92)
ACTL6A
SMARCA4
SMARCA2
Actin
B
ACTL6A
SMARCA4
SMARCA2
3381
176
208
25
39
145
299
C
ACTL6A KO vs control
-log10 (p-val)
Log2 Fold Change
SMARCA4 KO vs control
-log10 (p-val)
Log2 Fold Change
SMARCA2 KO vs control
-log10 (p-val)
Log2 Fold Change
D
Enrichment plot: GO_CHROMOSOME_ORGANIZATION
NES = 2.346
p-val = 0
Enrichment plot: GO_CONDENSED_CHROMOSOME
NES = 2.163
p-val = 0.004
Enrichment plot: GO_CHROMOSOMAL_REGION
NES = 2.115
p-val = 0.002
Enrichment plot: GO_CHROMATIN_ORGANIZATION
NES = 2.011
p-val = 0

Figure 4. Transcription regulation role of ACTL6A.

(A) Western blot analysis of HCT116 cells transduced with two indicated sgRNAs. RNA and protein samples were extracted day 7 post transduction. Actin served as a loading control.

(B) Venn diagram showing the overlaps between the differentially expressed genes in three knockout conditions. p-value <0.05, $|\log_2(\text{fold-change})|$ >0.5.

(C) Volcano plots of transcriptome sequencing data of ACTL6A, SMARCA4, and SMARCA2 knockout cells versus sgGFP control (n=3 for ACTL6A, n=2 for SMARCA4, n=1 for SMARCA2). Red dots indicate FDR values <0.05 and $|\log_2(\text{fold-change})|$ >1.

(D) GSEA performed on transcriptome sequencing data from ACTL6A depleted HCT116 cells. NES, normalized enrichment score.

ACTL6A is required for cell proliferation in both SMARCA4/2 proficient and deficient cancer cell line.

To further clarify the significance of SMARCA4/2 in ACTL6A-mediated cell proliferation, I utilized SMARCA4/2 proficient cells (AGS, HCT116), SMARCA4 deficient cells (A549, H1299), and SMARCA4/2 deficient cells (SNU484, H522) (Figure 5A). Immunofluorescence staining and immunoblot of nuclear and cytoplasmic fraction of SMARCA4/2^{Pro} and SMARC4/2^{Def} cell lines showed nuclear localization of ACTL6A regardless of SMARCA4/2 presence (Figures 5B and 5C). Loss of SMARCA4/2 functional subunit changes the SWI/SNF complex assembly. Previous study defines two types of SWI/SNF complex subunits upon SMARCA4/2 loss – intact residual complex and detached ATPase module subunits [4]. Unlike residual complex units such as SMARCC1 and SMARCB1, ATPase module subunits ACTL6A and SS18 does not bind with SWI/SNF complex in SMARCA4/2 deficient cells (Figure 5D). Therefore, in SMARCA4/2^{Pro} cells, SWI/SNF complex bound ACTL6A localizes to nucleus. In SMARCA4/2^{Def} cells, unbound ACTL6A localizes to nucleus, further implying independent ACTL6A mechanisms. Depletion of ACTL6A in all three SMARCA4/2 conditions caused equally effective cell proliferation arrest (Figures 5D and 5E). In contrast, depletion of ARID1A had no effect on cell proliferation, consistent with previous findings that the loss-of-function mutation of ARID1A drives cancer [27]. This strengthens my claim that compared to other SWI/SNF complex subunits, ACTL6A has more essential role in cell growth. Most importantly, ACTL6A mediates gastrointestinal cancer cell proliferation regardless of SMARCA4/2 deficiency.

Fig.5

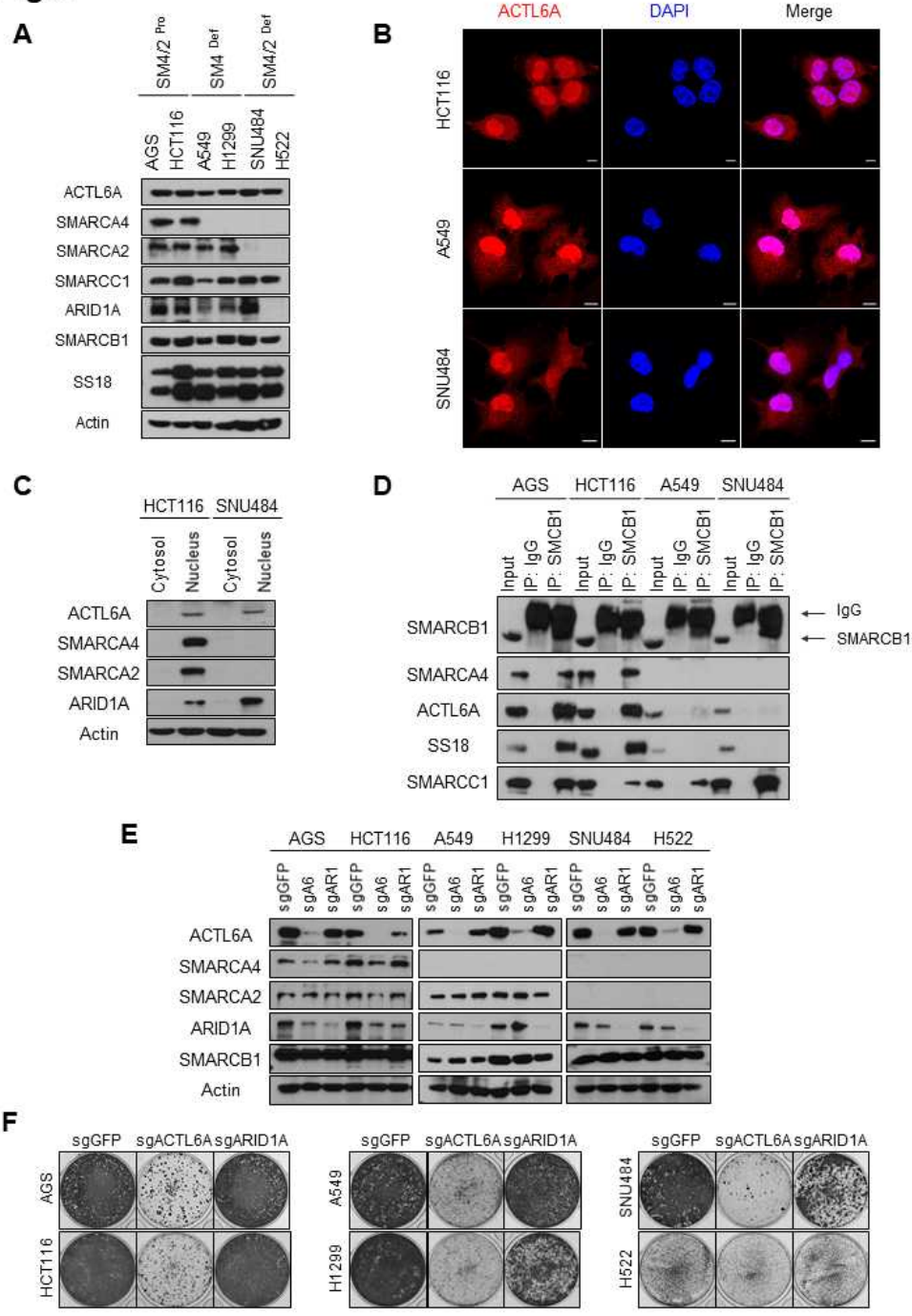
A
SM4/2 Pro
SM4 Def
SM4/2 Def
AGS
HCT116
A549
H1299
SNU484
H522
ACTL6A
SMARCA4
SMARCA2
SMARCC1
ARID1A
SMARCB1
SS18
Actin
B
ACTL6A
DAPI
Merge
HCT116
A549
SNU484
C
HCT116
SNU484
Cytosol
Nucleus
ACTL6A
SMARCA4
SMARCA2
ARID1A
Actin
D
AGS
HCT116
A549
SNU484
Input
IP: IgG
IP: SMCB1
IgG
SMARCB1
SMARCA4
ACTL6A
SS18
SMARCC1
E
AGS
HCT116
A549
H1299
SNU484
H522
sgGFP
sgA6
sgAR1
ACTL6A
SMARCA4
SMARCA2
ARID1A
SMARCB1
Actin
F
sgGFP
sgACTL6A
sgARID1A
AGS
HCT116
A549
H1299
SNU484
H522

Figure 5. Localization, SWI/SNF complex formation, and depletion of ACTL6A in SMARCA4/2 proficient and deficient cell lines.

(A) Western blot analysis of SMARCA4/2 proficient, SMARCA4 deficient, and SMARCA4/2 deficient cell lines; AGS (gastric), HCT116 (colon), A549 (lung), H1299 (lung), SNU484 (gastric). Actin served as a loading control.

(B) Immunofluorescence assay of HCT116, A549, and SNU484 cell lines. ACTL6A (red), nuclear staining by DAPI (blue) and merged images on right. Scale bar = 10μM.

(C) Western blot analysis of cytosoplasmic and nuclear fraction of HCT116 and SNU484.

(D) Immunoprecipitation using SMARCB1 in SMARCA4/2^{Pro} and SMARC4/2^{Def} cell lines. Arrows indicate IgG and SMARCB1 accordingly.

(E) Western blot analysis of cell lines transduced with indicated sgRNAs, where sgA6 represent ACTL6A sgRNA (4+5) and sgAR1 represent ARID1A sgRNA (1+2).

(F) Colony formation assay after depletion of ACTL6A or ARID1A.

ACTL6A induce genome-wide histone enrichment modification in SMARCA4/2 proficient and deficient cell lines.

To determine the mechanisms of ACTL6A-mediated transcription regulation, ACTL6A depletion mediated genome-wide histone enrichment modifications were analyzed. First, chromatin immunoprecipitation (ChIP) sequecing of active histone marker H3K27ac was conducted using SMARCA4/2^{Pro} HCT116, and SMARCA4/2^{Def} SNU484 cell lines. Loss of ACTL6A resulted in genome wide decrease of H3K27ac enrichment in both cell lines (Figure 6A).

Decreased H3K27Ac enrichment was more significant in promoter sites than enhancer sites (Figure 6B). Occupancy of SMARCA4 has been reported in distal regulatory elements, including enhancers [4]. This indicates that ACTL6A targets and regulates transcription separately from SMARCA4. Collectively, ACTL6A modifies genome-wide histone enrichments, which consequently regulate global gene expression and cell proliferation in gastrointestinal cancer.

Fig.6

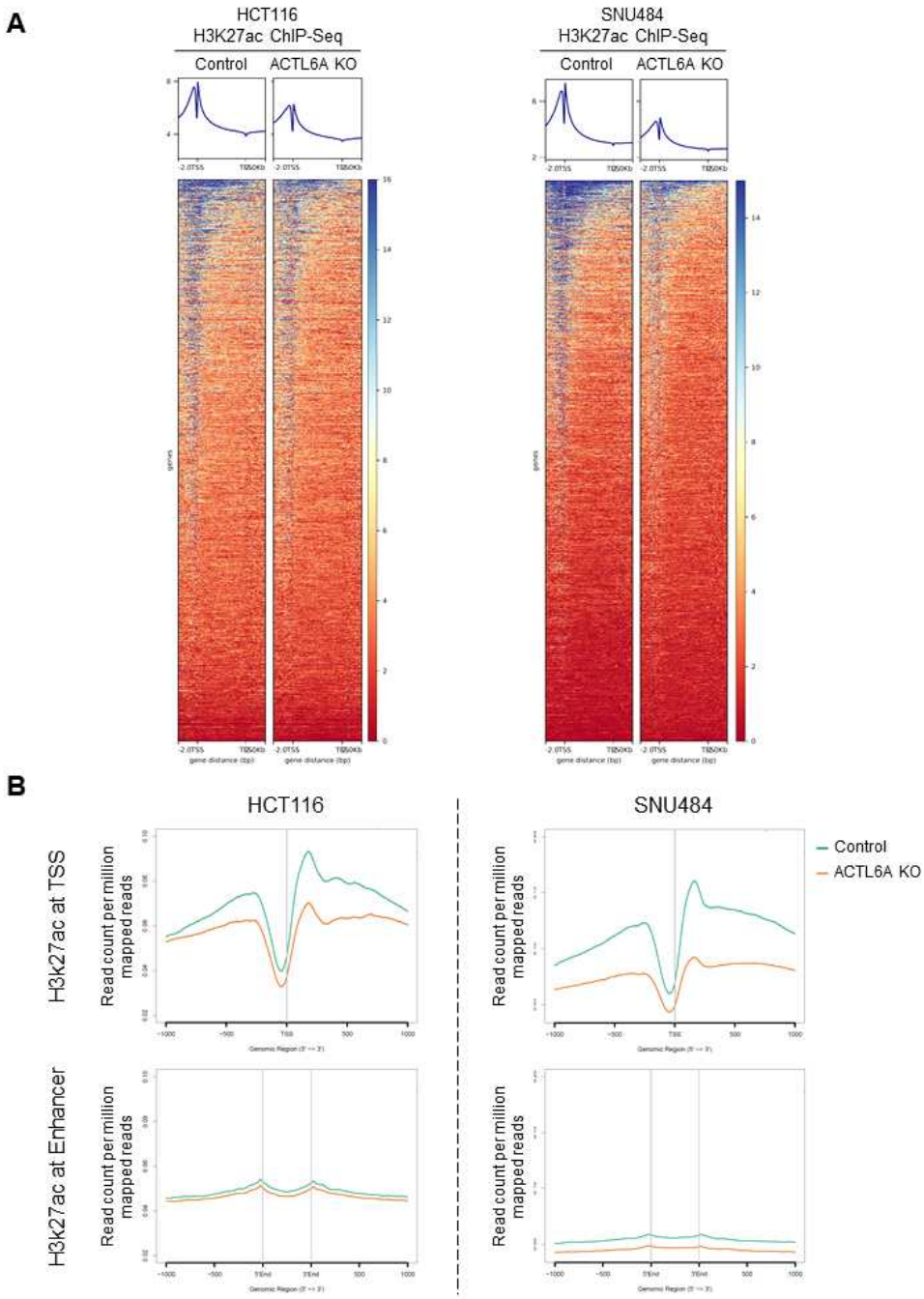
A
HCT116
H3K27ac ChIP-Seq
Control
ACTL6A KO
SNU484
H3K27ac ChIP-Seq
Control
ACTL6A KO
B
HCT116
SNU484
H3k27ac at TSS
H3k27ac at Enhancer
Read count per million mapped reads
Control
ACTL6A KO

Figure 6. Genome-wide histone enrichment modification of ACTL6A.

(A) Heatmaps showing H3K27ac enrichment in HCT116 & SNU484, transduced with control sgGFP or sgACTL6A. Rows corresponds to ±2-kb region from transcriptional start sites (TSS) to transcriptional end sites (TES) for each gene.

(B) Metaplots showing H3K27ac signal at TSS or enhancer sites in control (green) and ACTL6A KO (orange) in HCT116 and SNU484.

Discussion

Through functional studies and epigenetic profiling, I confirmed that ACTL6A is an essential transcription factor in gastrointestinal cancer. I aimed to identify the molecular mechanism of ACTL6A. Previous study using CRISPR/Cas9 library screening identified ACTL6A as an essential epigenetic modifier in gastrointestinal cancer.

Since the previous study used dual sgRNA-guided CRISPR/Cas9 system to conduct loss-of-function study for ACTL6A, verification studies to ensure specificity were conducted to eliminate the possibility of any non-specific, off-target effects of CRISPR/Cas9 system [28]. Consequently, loss-of-function tests were performed with two different single sgRNA-guided CRISPR/Cas9 system, as well as shRNA-mediated inhibition. Depletion of ACTL6A effectively induced cell proliferation. Further validation was conducted using CRISPR/Cas9-resistant clone of ACTL6A in a reversibility assay. Introduction of CRISPR/Cas9-resistant clone mitigated the effects of ACTL6A inhibition. Taken together, I confirmed that the loss of ACTL6A induce apopotosis and cell proliferation inhibition in gastrointestinal cancer.

Functional studies of ACTL6A in relation to SWI/SNF complex indicates that depletion of ACTL6A is more effective in cell proliferation inhibition compared to other SWI/SNF subunits such as SMARCA4, SMARCA2, and ARID1A. ARID1A is known to be most frequently mutated among the SWI/SNF complex subunits [29]. Therefore, the comparison of ACTL6A and

ARID1A depletion clarifies that the cell proliferation inhibiting effects of ACTL6A does not pertain to all SWI/SNF complex subunits. My results imply that ACTL6A is an essential and critical SWI/SNF complex subunit. I demonstrated that SWI/SNF complex subunit ACTL6A regulates protein stability of catalytic subunits SMARCA4/2 in gastrointestinal cancer. GSEA of ACTL6A DEGs showed enrichment in chromosomal organization, which was expected of a SWI/SNF complex subunit. To confirm that ACTL6A regulate transcription via SWI/SNF complex modification, transcriptome sequencing was conducted. However, RNA-seq of ACTL6A, SMARCA4, and SMARCA2 knockout cells showed minimum overlap between DEGs of three subunits.

In order to test the SWI/SNF complex dependent and independent functions of ACTL6A, SMARCA4/2 proficient and decificient cell lines were used. ACTL6A expression and nuclear localization was independent of SMARCA4/2. According to a recent study on SWI/SNF complex assembly, loss of SMARCA4/2 functional subunit results in intact catalytic activity-independent complex as well as detached catalytic activity-dependent subunits [4]. As a known catalytic activity-dependent subunit, ACTL6A does not interact with SWI/SNF complex in SMARCA4/2 deficient cells. Interestingly, depletion of ACTL6A in both SMARCA4/2 proficient and deficient cell lines effectively inhibited cell proliferation. These results collectively indicate that while ACTL6A shares the chromatin remodeling role of SWI/SNF complex, ACTL6A also functions independently to regulate transcription and cell proliferation.

ChIP-seq using histone activation marker H3K27Ac was conducted in

SMARCA4/2^{Pro} HCT116 and SMARCA4/2^{Def} SNU484 cell lines, after ACTL6A depletion. Loss of ACTL6A induced loss of genome-wide H3K27Ac enrichment in both cell types. Transcriptome analysis of ACTL6A-depleted HCT116 and SNU484 showed similar ratio of up-regulated and down-regulated DEGs, which implies that ACTL6A can activate and repress transcription. Additional ChIP-seq using histone repressive marker H3K27me3 could provide more comprehensive ACTL6A mediated histone modifications.

While ACTL6A binds to both promoter and enhancer sites, ACTL6A-depleted cells showed more significant H3K27Ac enrichment loss in promoter sites in both cell lines. However, SMARCA4/2 is known to bind preferentially to enhancer sites. Further study of epigenetic mechanisms of ACTL6A in SMARCA4/2 proficient and deficient cancer cell can specify SMARCA4/2 independent role of ACTL6A. Motif analysis study will allow identification of specific transcription factors recruited or regulated by ACTL6A. Identification of ACTL6A-regulated transcription factors integrated with transcriptome, ATAC-seq, and ChIP assay results will present more comprehensive analysis of SMARCA4/2 dependent and independent ACTL6A functions.

Due to the limited understanding of cancer specific function of ACTL6A, no specific therapies are currently available. Current therapeutic options that target SWI/SNF complex focus on subunit specific vulnerabilities [1,6]. Although the possibility of clinical implication needs to be further defined and tested, identification of SWI/SNF independent, transcription regulating

role of ACTL6A could provide a novel insight and mechanism on targeting chromatin remodeling complex subunits. In conclusion, my data presents that ACTL6A regulates genome-wide histone modifications and global gene transcription.

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prognosis of hepatocellular carcinoma and promotes metastasis and epithelial-mesenchymal transition. Hepatology 2016; 63:1256-1271.

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국문초록

SWI/SNF 크로마틴 리모델링 복합체는 인간에게 발생하는 암의 20% 이상에서 변이되어 있다고 알려져 있다. SWI/SNF 복합체를 이루고 있는 다양한 단백질 유닛들의 분자 기전에 관한 연구는 활발히 진행되어 왔는데, 이는 암에서 각 SWI/SNF 복합체 유닛들의 크로마틴 구조 및 유전자 발현 조절 메커니즘을 규명함으로써 특이적이고 새로운 치료 전략을 세울 수 있다고 예측되기 때문이다. BAF53a 라고도 알려진 액틴 유사 단백질 ACTL6A는 SWI/SNF 크로마틴 리모델링 복합체를 이루는 유닛 중 하나로 알려져 있다. 이전 연구에서 CRISPR/Cas9 knockout 스크리닝을 통해 위암의 세포 성장에 영향을 미치는 후성유전학 변형인자인 ACTL6A를 발견하였다. 그러나, ACTL6A가 위장관암 증식에 관여하는 역할이나 메커니즘은 아직 보고된 바가 없다. 그렇기에 본 연구에서는 후성유전학 변형인자인 ACTL6A가 SWI/SNF 복합체의 일부로서 유전자를 조절하는 분자 기전을 규명하고자 한다.

먼저, CRISPR/Cas9과 shRNA 로 ACTL6A를 억제하면 위장관암의 세포 성장이 감소하였으며, 이때 CRISPR/Cas9 resistant 한 ACTL6A를 주입하면, 세포 성장이 다시 회복되었다. 마우스 생체 모델에서 ACTL6A를 억제했을 때에도 종양 성장이 억제되는 것을 확인함으로써 ACTL6A가 위장관암에서 세포 성장을 조율하는 역할이 있음을 확인하였다.

SWI/SNF 복합체와 관련된 ACTL6A의 역할을 확인해 본 결과, SMARCA4, SMARCA2, 및 ARID1A 등의 다른 SWI/SNF 복합체 유닛들을 억제했을 때 보다, ACTL6A 억제 시 위장관암의 세포 성장이 더 효과적으

로 감소하였다. 또한, ACTL6A는 SWI/SNF 복합체의 ATP 촉매 핵심 단백질인 SMARCA4/2의 단백질 안정성을 조절하였다. ACTL6A의 세포핵 내의 발현은 SMARCA4/2를 보유한 세포주와 보유하지 않은 세포주에서 동일하였다. 기존에 알려진 대로, SMARCA4/2가 없는 세포주에서 ACTL6A는 SWI/SNF 복합체와 구성체를 이루고 있지 않았다. 추가로, ACTL6A의 역할을 SMARCA4/2를 보유한 세포주와 보유하지 않은 세포주에서 확인해본 결과, SMARCA4/2 유무와 관계없이 ACTL6A 억제 시 세포 성장이 효과적으로 감소하였다. 즉, ACTL6A는 SWI/SNF 복합체와 독립적인 전사 조절 기전도 보유한다고 예측할 수 있다.

ACTL6A가 불활성화된 세포들의 전사체를 분석해본 결과, ACTL6A가 염색체 리모델링을 통해 전반적인 전사 조절을 한다는 것을 유추할 수 있었다. ACTL6A가 불활성화된 세포주를 사용해 ChIP-seq을 진행해본 결과, 히스톤 활성화의 지표인 H3K27Ac의 결합이 감소하였다. 이 결과들을 종합해본 결과, ACTL6A는 위장관암에서 유전자 전반적인 히스톤 변형을 일으킴으로써 유전자 발현 및 전사를 한다는 것을 확인하였다.

주요어: ACTL6A/BAF53a/Arp4; SWI/SNF complex; SMARCA4/Brg1; SMARCA2/BRM; Histone modification; Transcription regulation; Epigenetics; Gastrointestinal cancer;

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