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이학석사 학위논문

Epigenetic modifier ACTL6A regulates transcription through mSWI/SNF and INO80 complex in gastrointestinal cancer

위장관암에서 mSWI/SNF 와
INO80 복합체를 통한 후성유전학적 변형인자
ACTL6A 의 전사 조절 기전

February 2023

서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과

양 혜 주

위장관암에서 mSWI/SNF와 INO80 복합체를 통한 후성유전학적 변형인자 ACTL6A의 전사 조절 기전

지도교수 김 태 유

이 논문을 이학석사 학위논문으로 제출함

2023년 2월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과

양 혜 주

양 혜 주의 이학석사 학위논문을 인준함

2023년 2월

위 원 장 _____ 강 경 훈 (인)

부위원장 _____ 김 태 유 (인)

위 원 _____ 이 유 진 (인)

Epigenetic modifier ACTL6A regulates transcription through mSWI/SNF and INO80 complex in gastrointestinal cancer

by
Hye-Ju Yang
(Directed by Tae-You Kim, M.D., Ph.D.)

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

Department of Molecular Medicine and Biopharmaceutical Sciences,
World Class University Graduate School of Convergence Science
and Technology, Seoul National University
February 2023

Approved by thesis committee:

Chair GyeongHoon Kang (Seal)

Vice Chair TaeYou Kim (Seal)

Examiner Eugene C. Yi (Seal)

Abstract

Epigenetic modifier ACTL6A regulates transcription through mSWI/SNF and INO80 complex in gastrointestinal cancer

Hye-Ju Yang

Department of Molecular Medicine

And Biopharmaceutical Sciences

World Class University Graduate School

Of Convergence Science and Technology

Seoul National University

Chromatin remodeling complexes can regulate the chromatin accessibility and structure of genomic regions where they reside to affect the binding of transcription factors and thereby control target gene expression [19]. The mammalian SWI/SNF complex plays a role in ATP-dependent chromatin remodeling and the molecular mechanism of SWI/SNF subunits has been studied, because 25% of cancers are caused by mutation of the subunits [20,29]. The INO80 complex is another ATP-dependent chromatin remodeling complex, which functions in transcription and DNA repair. INO80 subunits were frequently present in high copy numbers and exhibited a high rate of amplification and increased protein expression in colon cancer [13]. ACTL6A is one of the subunits of the SWI/SNF complex and the INO80 complex.

In a previous study, CRISPR-Cas9 knockout screening identified ACTL6A, an epigenetic modifier that plays a role in gastric cancer cell growth. However, the mechanism of ACTL6A involved in gastrointestinal cancer proliferation has not yet been accurately reported. Therefore, this study aims to identify the molecular mechanism of ACTL6A related to the SWI/SNF and INO80 complex in gastrointestinal cancer.

ACTL6A interacts with SWI/SNF complex and INO80 independently. Genome wide SMARCB1 enrichment was increased upon ACTL6A depletion. Furthermore, INO80 occupancy was reduced after loss of ACTL6A. As a result of motif analysis through SMARCB1 and INO80 ChIP-seq of ACTL6A depletion cells,

SWI/SNF and INO80 complex regulate the chromatin state of the AP-1 motif through ACTL6A.

Together, ACTL6A is required for proper chromatin organization through interaction with the INO80 complex as well as the SWI/SNF complex. Altered occupancy of these two complexes lead to changes in chromatin accessibility and histone modification, and eventually to changes in gene expression. My findings may provide a potential treatment strategy by targeting ACTL6A, a subunit of the mammalian SWI/SNF and INO80 chromatin remodeling complex.

Keywords: ACTL6A; SWI/SNF complex; INO80 complex; AP-1; Histone modification; Chromatin remodeling; Gastrointestinal cancer; Epigenetics; Transcription regulation;

Student number: 2021-20896

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Introduction

Epigenetic changes such as aberrant DNA methylation, histone modifications, and chromatin regulation are associated with tumorigenesis. Also, epigenetic regulators have critical roles in cancer development [1-3]. Among epigenetic factors, the ATP-dependent chromatin remodeling complexes can alter nucleosome composition and position to influence chromatin structure and gene expression [4,5]. The enzymatic remodeling activity allows DNA binding proteins like transcription factors to access their binding sites for transcription regulation. In addition, by controlling the packing and unpacking of the chromatin, chromatin remodelers provide regulated DNA accessibility and have roles in cell fate decision during cancer development and progression [6].

ATP-dependent chromatin remodelers can be divided into four families based on subunit compositions and domain structures, including SWI/SNF(BAF), INO80, ISWI and CHD [7]. SWI/SNF complex is composed of approximately 15 protein subunits including SMARCA4/SMARCA2 ATPase subunit, SMARCB1, and SMARCC1 [8]. Recent studies showed that mutations in the SWI/SNF subunits are associated in various human cancers [9]. Mutations found on the ARID1A gene lead to loss of function of ARID1A, which occurs in ~6% of cancers, and functional impairment of ARID1A can be resistant to anti-EGFR therapies in colorectal tumors. [10,11]. SMARCB1 is

inactivated by biallelic mutations in nearly all cases of rhabdoid tumor [12].

INO80 complex functions in many chromosomal processes that are crucial for genome stability and modulates chromatin structure by mobilizing nucleosomes through ATP hydrolysis [15]. INO80 subunits have a high rate of amplification in many human cancer types. Previous study showed that haploinsufficient mutation of INO80, the catalytic ATPase of the INO80 complex, inhibits intestinal tumors in mouse model of colon cancer [13]. In lung cancer, INO80 complex promotes oncogenic transcription and tumor growth [14].

Actin-like 6A (ACTL6A), known as BAF53a/INO80K/ARP4, is a subunit of SWI/SNF(BAF) and INO80 complex. It is crucial for the maintenance of stem or progenitor cells during mammalian embryonic development. ACTL6A is known to be upregulated in various cancers, including colon cancer, osteosarcoma, and glioma cells [18] and associates with poor prognosis. However, molecular mechanism of ACTL6A in gastrointestinal cancer is not well characterized. In addition, the role ACTL6A plays in SWI/SNF and INO80 complex is less clear.

In this functional study of ACTL6A, I investigate the molecular mechanism of ACTL6A relative to mammalian SWI/SNF and INO80 chromatin remodeling complex. Epigenetic modifier ACTL6A is essential for cell proliferation, and ACTL6A depletion leads to widespread decrease in global H3K27ac levels and chromatin accessibility changes. Upon ACTL6A loss, altered occupancy of

these two complexes lead to changes in chromatin accessibility and histone modification, and eventually to changes in gene expression, which affects the regulation of cell proliferation. The SWI/SNF and INO80 complexes occupy the AP-1 (Activator protein-1) motif, which in turn regulates gene expression as chromatin status changes. My study defines a critical role of ACTL6A in proper chromatin organization through interaction with the INO80 complex as well as the SWI/SNF complex. Overexpression of ACTL6A can modify transcription of ACTL6A-related genes by misregulation of the SWI/SNF and INO80 complex at AP-1 motif in gastrointestinal cancer.

Materials and Methods

Cell culture & reagents

Cells were obtained from ATCC (American Tissue Culture Collection) or the Korean cell bank. Cells were regularly tested for mycoplasma contamination. Cells were cultured in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum (FBS) and gentamicin (10 μg/mL) at 37 °C in a humidified 5% CO_2 atmosphere.

Constructs

pBABE-puro (Addgene, 1764) backbone was used for overexpression cloning. GFP-SMARCA4 (Addgene, 65391) plasmid was used for SMARCA4 overexpression.

Colony formation assay (CFA)

Colony formation assay was performed in 6 well plates. Knockout system transduced cells were seeded in duplicate. Six to ten days later, the media was removed, and the cells were stained with Coomassie Brilliant Blue solution (0.1% Coomassie Brilliant Blue R-250, 10% acetic acid, 50% methanol) for 5 hours at room temperature. Cells were washed two times in PBS (phosphate buffer saline).

Immunoprecipitation

Cells were incubated at 4 °C for 30 min in a lysis buffer (50mM Tris-HCl (pH 7.5), 1% NP-40, 0,1% sodium deoxycholate, 150mM NaCl, 1mM sodium pyrophosphate, and protease/ phosphatase inhibitors). Whole cell extract was obtained after centrifugation at 13,000 rpm for 20 min, and incubated with 5 μg antibodies in 4 °C for overnight. Whole cell extracts were incubated with Dynabead (Invitrogen, #10004D, #10002D) in 4 °C for 4 hours.

Antibodies to the following were used: Rabbit IgG (Abcam, ab37415); Mouse IgG (Abcam, ab37355); SMARCB1 (Bethyl, #A301-087A); SMARCC1 (Santa Cruz, #sc32793); INO80 (Abcam, #118787).

Virus production and transduction

The LentiCRISPRv2 vector system was obtained from Addgene (#52961). Two distinct sgRNAs were designed for each gene. Lentiviruses were produced by transducing 293FT cells with lentiCRISPRv2 plasmid using Virapower packaging mix (Invitrogen). The viruses were harvested and target cells were transduced with viruses in the presence of 6 μg/ml polybrene (Sigma). After 24 hours, the transduced cells were cultured with 1 μg/ml puromycin (Sigma) for 6 days. Expression changes of target gene was validated by western analysis. The oligo sequences used for sgRNA are noted in Table 1.

Table 1. Primer sequence for sgRNA

Gene	(Sequence 5′ –> 3′)	
ACTL6A-4	F	caccgGTTGAAGGACATAGCCATCG
ACTL6A-4	R	aaacCGATGGCTATGTCCTTCAACc
ACTL6A-5	F	caccgTGCCAAGACCTCGTAACCTG
ACTL6A-5	R	aaacCAGGTTACGAGGTCTTGGCAc
SMARCA4-1	F	caccgCTGGCCGAGGAGTTCCGCCC
SMARCA4-1	R	aaacGACCGGCTCCTCAAGGCGGGc
SMARCA4-3	F	caccgCCTGTTGCGGACACCGAGGG
SMARCA4-3	R	aaacGGACAACGCCTGTGGCTCCCc
FOS-2	F	caccgGGCGTTGTGAAGACCATGAC
FOS-2	R	aaacGTCATGGTCTTCACAACGCCc
JUN-4	F	caccgGATTATCAGGCGCTCCAGCT
JUN-4	R	aaacAGCTGGAGCGCCTGATAATCc

Western blot analysis

Cells were harvested and washed twice with PBS. The cells were then incubated at 4 °C for 30 min in a lysis buffer (50mM Tris-HCl (pH 7.5), 1% NP-40, 0,1% sodium deoxycholate, 150mM NaCl, 1mM sodium pyrophosphate, and protease/ phosphatase inhibitors). After centrifugation at 13,000 rpm for 20 min, lysates were collected. Protein concentrations were quantified with BSA (Bicinchoninic Acid Protein Assay Reagent (Pierce, #23225)), according to manufacturer's instructions. The same amount of protein (1 μg/μL) was run in a 8~12% SDS-PAGE gel and transferred onto a Nitrocellulose membrane. Blocking was done in 5% skim milk solution. Blots were incubated with the primary antibody at 4 °C for overnight. Blots were incubated with an HRP-conjugated secondary antibody for 1 hours at room temperature. Proteins signals were detected using the ECL (enhanced chemiluminescent substrate).

Antibodies

Antobodies for the following were used in this study :

ACTL6A (Bethyl, #A301-391A); ACTL6A (Santa Cruz, #sc137062); β-Actin (Santa Cruz, #sc47773); HA (Abcam, #9110); SMARCA4 (Abcam, #110641); SMARCA2 (Cell signaling Technologies, #11966); SMARCC1 (Santa Cruz, #sc32793); SMARCB1 (Bethyl, #A301-087A); SS18 (Cell Signaling Technologies, #21792); INO80 (Abcam, #118787); YY1 (Santa

Cruz, #sc7341); c-Fos (Abcam, #222699); c-Jun (Cell Signaling Technologies, #9165)

ATP assay

Organoids were seeded in 24 well micro-assay-plates, at 5,000 cells /50 μL / well (n=4). ATP content was measured in accordance to the protocol of the CellTiter-Glo 3D cell viability assay kit (Promega #G9682).

Chromatin immunoprecipitation (ChIP) assay and ChIP-seq

ChIP assay and ChIP-seq were performed as previously described [32, 22].

ATAC (Assay for Transposase-Accessible Chromatin) and ATAC-seq

To generate ATAC libraries, 1 x 10^5 cells were harvested, treated with digitonin and tagmented with 2.5 μL Tn5 Transposase for 30 min. DNA was purified using DNA Clean & Concentrator-5 kit (Zymo research) and amplified up to 15 cycles. Libraries were diluted and sequenced on Illumina NextSeq at paired-end reads.

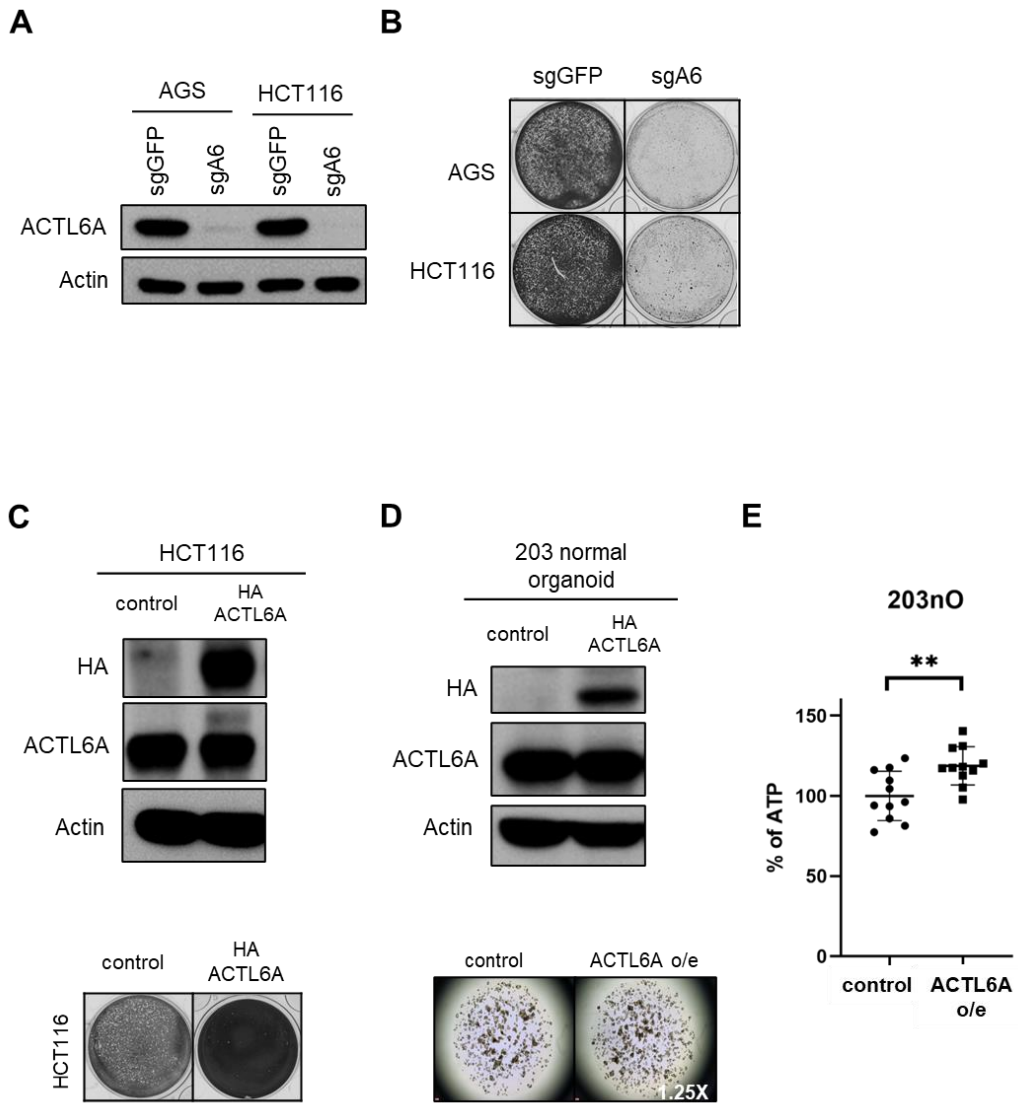
Results

ACTL6A is essential for cell survival and proliferation in GC and CRC cells

In previous studies, CRISPR-Cas9 knockout screening identified ACTL6A, an epigenetic modifier in gastric and colon cancer, and observed that the expression level of ACTL6A was upregulated in gastric and colon cancer cell lines [21]. ShRNA-mediated ACTL6A knockdown also inhibited cell growth in GC and CRC cells. It was confirmed that ACTL6A depletion caused apoptosis in gastric and colon cancer cell lines through an increase in apoptosis related protein and Annexin V staining. Likewise, in the *in vivo* study, ACLT6A affected tumor proliferation. [22].

To prove the growth effects of ACTL6A, ACTL6A loss-of-function and gain-of-function analyses were conducted. CRISPR-Cas9 approach with sgRNAs targeting ACTL6A resulted in decreased cell proliferation in AGS and HCT116 cell lines. The colony forming assay showed that formed fewer colonies than the control cells. (Figure 1A and 1B). Next, overexpressing ACTL6A in HCT116 showed significant increased proliferation compared with control cells (Figure 1C). Even the same experiment was performed on patient-derived normal organoid, the growth rate was increased (Figure 1D and 1E). These results indicate that ACTL6A regulates proliferation in gastrointestinal cells.

Fig. 1


A
AGS
HCT116
sgGFP
sgA6
sgGFP
sgA6
ACTL6A
Actin
B
sgGFP
sgA6
AGS
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HCT116
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HA ACTL6A
HA
ACTL6A
Actin
control
HA ACTL6A
HCT116
D
203 normal organoid
control
HA ACTL6A
HA
ACTL6A
Actin
control
ACTL6A o/e
1.25X
E
203nO
**
% of ATP
150
100
50
0
control
ACTL6A o/e

Figure 1. ACTL6A is essential for cell survival and proliferation in GC and CRC cells

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

(B) Colony forming assay of AGS and HCT116 cells after ACTL6A depletion.

(C) Western blot analysis and colony forming assay of HA-tagged and endogenous ACTL6A expression in HCT116 cells. Actin was used as a loading control.

(D) Western blot analysis and cell growth of HA-tagged and endogenous ACTL6A expression in patient-derived normal organoid. Actin was used as a loading control.

(E) Proliferation of patient-derived organoid overexpressing ACTL6A or vector control by ATP assay

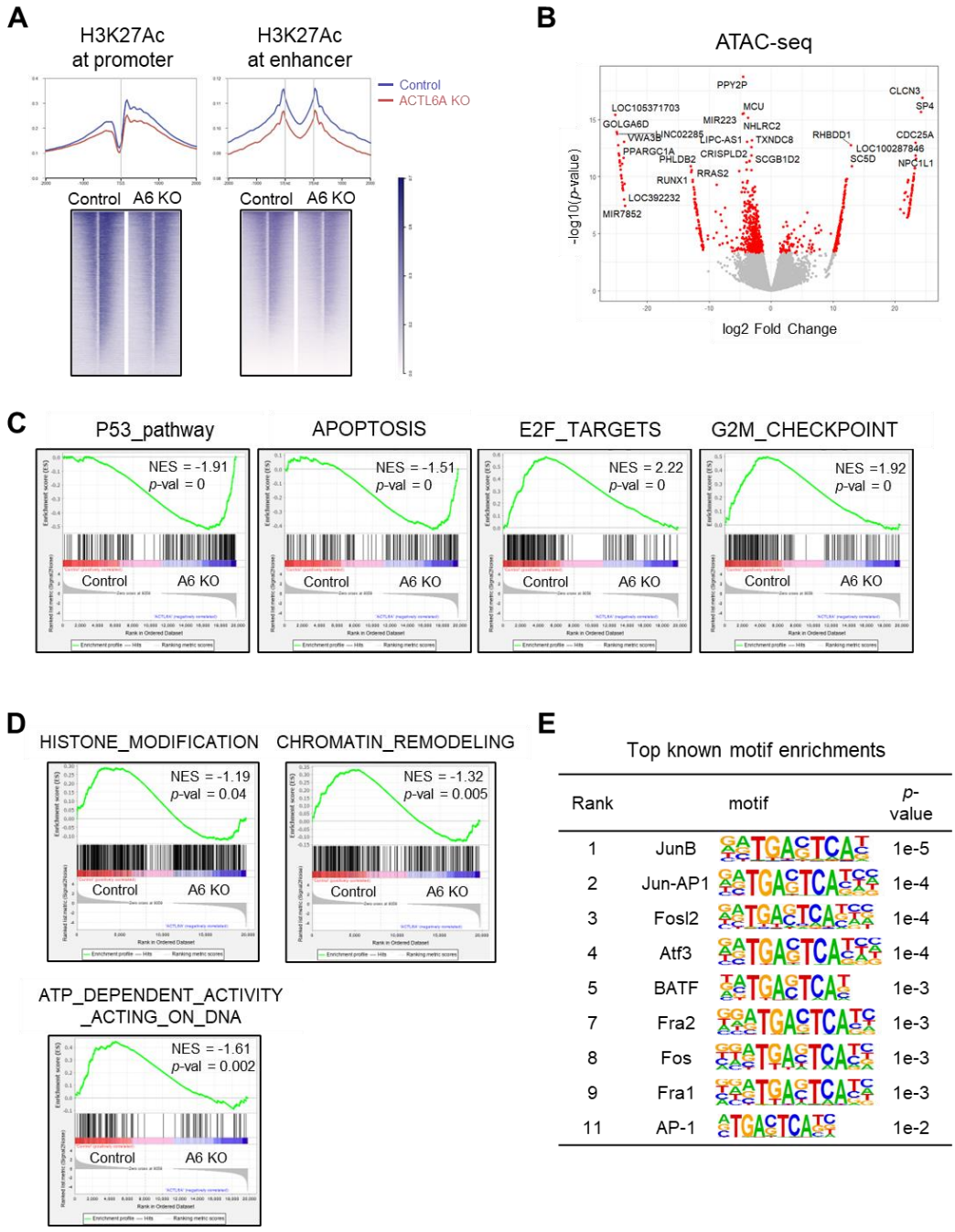
Loss of ACTL6A leads to global histone modification and changes in chromatin accessibility

To examine whether ACTL6A may influence chromatin status, ChIP-seq (Chromatin ImmunoPrecipitation sequencing) was conducted using active histone marker H3K27ac. Loss of ACTL6A resulted in genome wide decrease of H3K27ac enrichment (Figure 2A). To further examine the functional consequences of the loss of ACTL6A on chromatin accessibility, ATAC-seq (Assay for Transposase-Accessible Chromatin with sequencing) was performed in HCT116 cells before and after disruption of ACTL6A. A marked global change was observed in chromatin accessibility after ACTL6A depletion, supporting that the disruption of ACTL6A is accompanied by dramatic changes in chromatin structure (Figure 2B).

In a previous study, whole transcriptome sequencing showed that the depletion of ACTL6A resulted in about 1,000 differentially expressed compared with ACTL6A proficient HCT116 cells [22]. Gene set enrichment analysis (GSEA) of the DEGs of ACTL6A depletion cells showed enrichment in "p53 pathway", "apoptosis", and cell cycle-related pathways including "E2F targets" and "G2M checkpoint" (Figure 2C). In addition, "histone modification", "chromatin remodeling", and "ATP-dependent activity acting on DNA" were specifically downregulated in ACTL6A loss HCT116 cells, which were corresponds with ATP-dependent chromatin remodeler function (Figure 2D). Motif analysis was performed on ACTL6A depletion HCT116 cells to identify the

target of ACTL6A. Common motifs in H3K27ac ChIP-seq and ATAC-seq were AP-1 family members such as Fos, JunB, Fosl1/2, BATF, and ATF3 (Figure 2E). This suggests that ACTL6A not only plays a role in cell survival and growth, but also regulates gene expression by regulating histone modification and chromatin accessibility at genomic loci where AP-1 family members bind.

Fig. 2


A
H3K27Ac at promoter
H3K27Ac at enhancer
Control
ACTL6A KO
Control A6 KO
Control A6 KO
B
ATAC-seq
-log10(p-value)
log2 Fold Change
PPY2P
CLCN3
SP4
LOC105371703
MCU
GOLGA6D
MIR223
NHLRC2
VWA3B
LINC02285
LIPC-AS1
TXNDC8
RHBDD1
CDC25A
PPARGC1A
PHLDB2
CRISPLD2
SCGB1D2
LOC100287846
SC5D
NPC1L1
RUNX1
RRAS2
LOC392232
MIR7852
C
P53_pathway
NES = -1.91
p-val = 0
APOPTOSIS
NES = -1.51
p-val = 0
E2F_TARGETS
NES = 2.22
p-val = 0
G2M_CHECKPOINT
NES =1.92
p-val = 0
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A6 KO
D
HISTONE_MODIFICATION
NES = -1.19
p-val = 0.04
CHROMATIN_REMODELING
NES = -1.32
p-val = 0.005
ATP_DEPENDENT_ACTIVITY _ACTING_ON_DNA
NES = -1.61
p-val = 0.002
Control
A6 KO
E
Top known motif enrichments
Rank
motif
p-value
1 JunB 1e-5
2 Jun-AP1 1e-4
3 Fosl2 1e-4
4 Atf3 1e-4
5 BATF 1e-3
7 Fra2 1e-3
8 Fos 1e-3
9 Fra1 1e-3
11 AP-1 1e-2

Figure 2. Loss of ACTL6A leads to global histone modification and changes in chromatin accessibility

(A) Heatmap and metagene plot of H3K27ac ChIP-seq signal at TSS and enhancer region in control (blue) and ACTL6A depleted (red) in HCT116 cells. Rows correspond to ±2kb region from transcriptional start site to transcriptional end sites for each gene.

(B) Volcano plots of ATAC-seq data of ACTL6A knockout cells versus sgGFP control in HCT116. Red dots indicate FDR values<0.05, and | log2 fold change | >1.

(C), (D) Gene set enrichment analysis (GSEA) performed on transcriptome sequencing data from ACTL6A depleted HCT116 cells. NES, normalized enrichment score. (n=2)

(E) Top known motifs common in ATAC-seq and H3K27ac ChIP-seq after ACTLA depletion HCT116 cells identified by HOMER.

ACTL6A mediates gastrointestinal cancer cell proliferation regardless of SMARCA4/2 deficiency

ACTL6A is a known subunit of SWI/SNF complex and amplified in multiple cancers including head and neck squamous cell carcinoma, hepatocellular carcinoma, cervical cancer, and ovarian cancer [25–28]. Since previous studies have indicated that ACTL6A interacts with mutually exclusive ATPase subunit SMARCA4/2 [30], ACTL6A specific mechanism was analyzed using model systems of SMARCA4/2 proficient cells, SMARCA4 deficient cells, and SMARCA4/2 deficient cells (Figure 3A).

To clarify the significance of SMARCA4/2 in ACTL6A mediated cell proliferation, ACTL6A was depleted in all three SMARCA4/2 conditions and equally cell proliferation was arrested (Figure 3B and 3C). Also, SMARCA4 and/or SMARCA2 protein level was reduced after loss of ACTL6A (Figure 3B), supporting the previous study that ACTL6A destabilizes SMARCA4 [16]. Because the SWI/SNF complex is ATP-dependent, it requires the functional subunit SMARCA4 to catalyze ATP hydrolysis. Since depletion of ACTL6A destabilizes SMARCA4, the chromatin remodeling activity of the SWI/SNF complex could be modified. Therefore, to evaluate the contribution of SMARCA4 to ACTL6A-mediated transcriptional changes, SMARCA4 was depleted using CRISPR-Cas9 genomic editing. However, knockout of SMARCA4 itself did not have significant effect on gastrointestinal cancer cell proliferation (Figure 3D and 3E). SMARCA2 depletion also had the same effect in previous

study [22]. This suggests that ACTL6A mediated inhibition of cell proliferation and survival is independent of the presence of SMARCA4/2. Loss of ACTL6A inhibited cell proliferation equally in SMARCA4/2 proficient or deficient situations (Figure 3B and 3C). To validate these results, SMARCA4 re-expressing SMARCA4/2^{Def} SNU484 cells were modeled. Importantly, cell growth was significantly upregulated but reduction of ACTL6A still caused significant proliferative arrest in SMARCA4 expressing SNU484 cells (Figure 3D). These observations suggest that ACTL6A may generally affect global chromatin landscape through alternative mechanisms other than the regulation of SWI/SNF complex subunit occupancy within the genome. Therefore, ACTL6A-mediated cell proliferation and survival is independent of the presence of SMARCA4/2.

Fig. 3

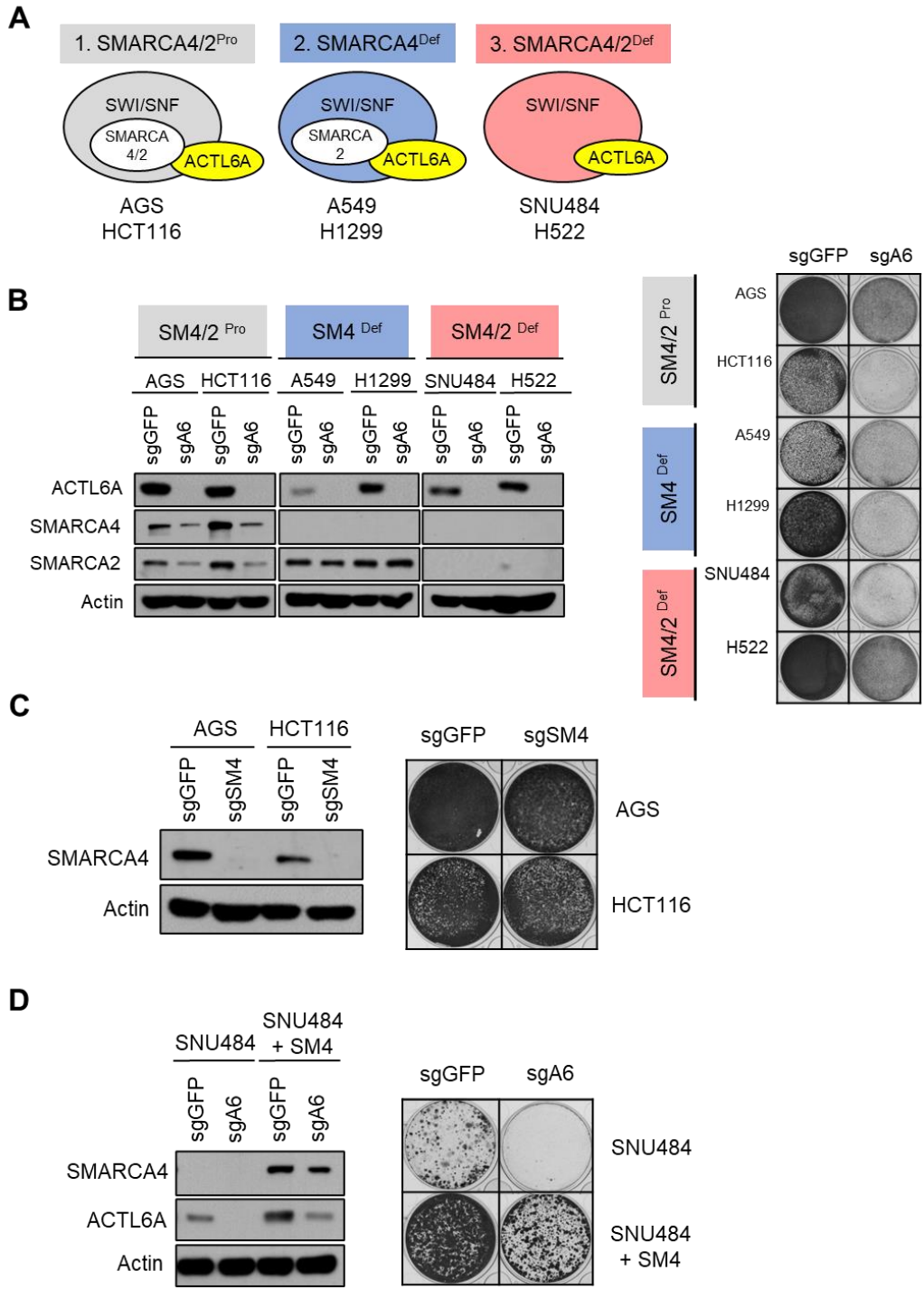

A
1. SMARCA4/2Pro
2. SMARCA4Def
3. SMARCA4/2Def
SWI/SNF
SMARCA 4/2
SMARCA 2
ACTL6A
AGS
HCT116
A549
H1299
SNU484
H522
B
SM4/2 Pro
SM4 Def
SM4/2 Def
AGS
HCT116
A549
H1299
SNU484
H522
sgGFP
sgA6
ACTL6A
SMARCA4
SMARCA2
Actin
sgGFP
sgA6
AGS
HCT116
A549
H1299
SNU484
H522
C
AGS
HCT116
sgGFP
sgSM4
SMARCA4
Actin
sgGFP
sgSM4
AGS
HCT116
D
SNU484
SNU484 + SM4
sgGFP
sgA6
SMARCA4
ACTL6A
Actin
sgGFP
sgA6
SNU484
SNU484 + SM4

Figure 3. ACTL6A mediates gastrointestinal cancer cell proliferation regardless of SMARCA4/2 deficiency

(A) Subunit composition of SMARCA4/2^{Pro}, SMARCA4^{Def}, SMARCA4/2^{Def} cells.

(B) Western blot analysis of SMARCA4/2^{Pro}, SMARCA4^{Def}, SMARCA4/2^{Def} cells transduced with indicated sgRNAs. Cell lines: AGS (gastric), HCT116 (colon), A549 (lung), H1299 (lung), SNU484 (gastric), H522 (lung). Actin was used as a loading control. Colony forming assay after depletion of ACTL6A in SMARCA4/2^{Pro}, SMARCA4^{Def}, SMARCA4/2^{Def} cells.

(C) Western blot analysis after depletion of SMARCA4 in SMARCA4/2^{Pro}, SMARCA4^{Def}, SMARCA4/2^{Def} cells. Whole cell extracts were prepared on day 7 post transduction. Actin was used as a loading control. Colony forming assay after depletion of SMARCA4 in SMARCA4/2^{Pro}, SMARCA4^{Def}, SMARCA4/2^{Def} cells.

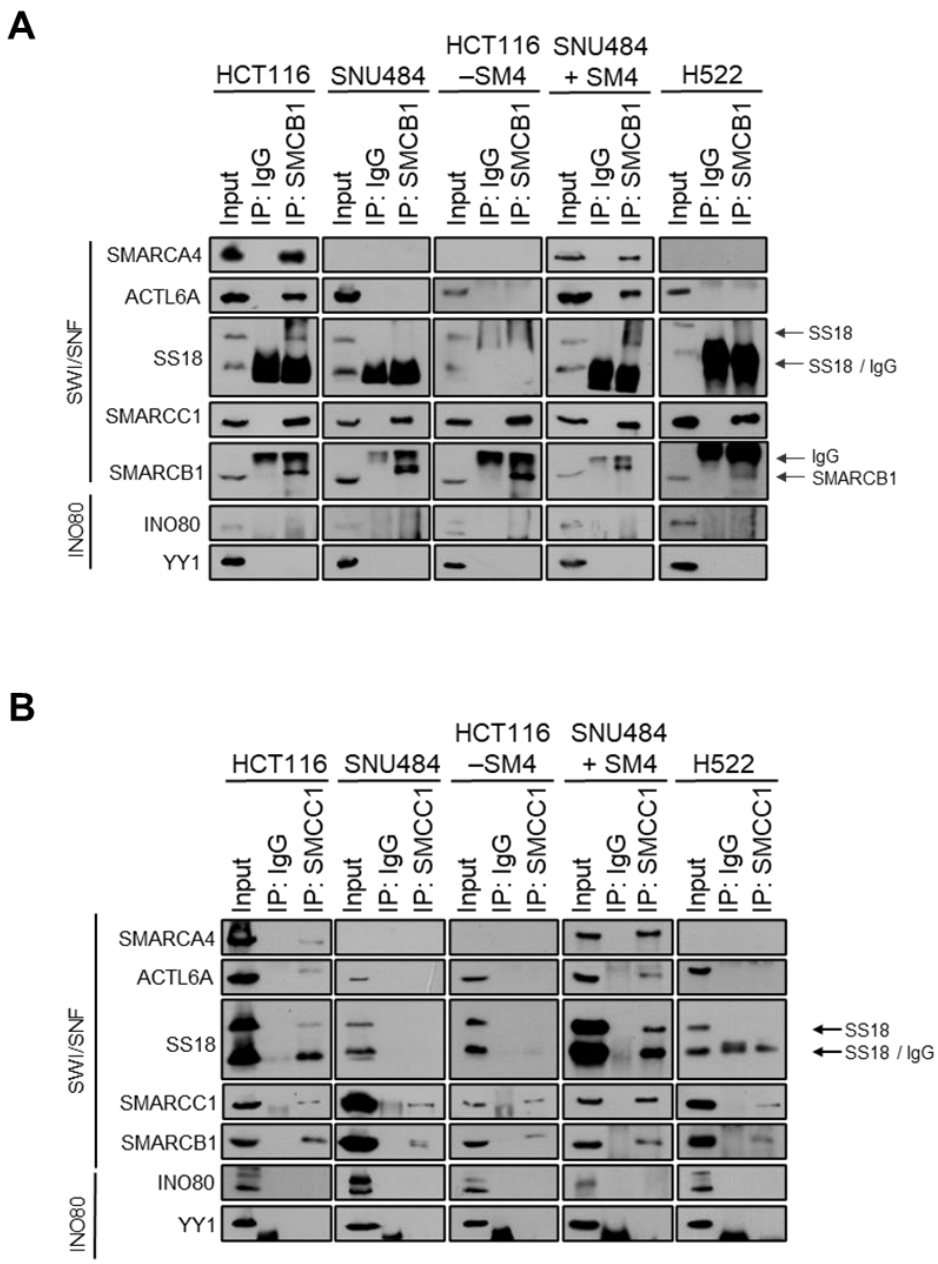
(D) Western blot analysis of SNU484 and SMARCA4-expressing SNU484 cells transduced with the indicated sgRNAs. Whole cell extracts were prepared on day 11 post transduction. Actin was used as a loading control. Colony forming assay of SNU484 and SMARCA4-expressing SNU484 cells transduced with the indicated sgRNAs.

ACTL6A is required for SWI/SNF-mediated chromatin organization

To confirm the endogenous interaction between ACTL6A and the SWI/SNF complex, co-immunoprecipitation (Co-IP) was conducted using HCT116, SNU484 and H522 basal cells. To investigate whether SMARCA4 plays a role in interaction with SWI/SNF and ACTL6A, I modeled SMARCA4 deficiency in HCT116 cells and SMARCA4-expressing in SNU484 cells and performed Co-IP. HCT116 cells were immunoprecipitated with antibody to SMRACB1 and ACTL6A and other known subunits of human SWI/SNF such as SMARCC1, SS18, and SMARCB1 were recovered. However, in SMARCA4-deficient HCT116 cells, SAMRCB1 was no longer associated with ACTL6A, SMARCA4 and SS18, while SMARCC1 was still present. In SMARCA4/2^{Def}cells (SNU484, H522), there was no interaction between SMARCB1 with ACTL6A, SMARCA4, SS18 while SMARCC1 still interacted. Interestingly, in SMARCA4-expressing SNU484 cells, ACTL6A and other SWI/SNF subunits were recovered by IP with antibody to SMARCB1. Meanwhile, the interaction of the SWI/SNF complex with INO80 and YY1 was not observed. indicating that the interaction is specific to SWI/SNF complex (Figure 4A). Co-IP with antibody SMARCC1 showed the same result (Figure 4B). These results are consistent with previous studies that ACTL6A and SS18 required the ATPase subunit SMARCA4/2 for SWI/SNF complex binding [31].

Next, to examine whether ACTL6A regulates genome wide histone modification via SWI/SNF complex, ChIP-seq data for H3K27ac and ATAC-seq data in ACTL6A depletion HCT116 cells were utilized. Active enhancer marker H3K27ac signal was reduced at SMARCA4 bound sites, suggesting that ACTL6A-dependent H3K27ac is consistent with SMARCA4 occupancy (Figure 4C). Also, chromatin accessibility was changed at SMARCA4 bound regions in ACTL6A deficient compared to ACTL6A proficient HCT116 cells. Decreased (n=5,137) and increased (n=4,088) accessible regions were identified with an average 2-fold change. (Figure 4D). Taken together, ACTL6A is required for establishing and maintaining genome-wide chromatin organization through interaction with SMARCA4 to the targeting of SWI/SNF complexes on chromosome. Next, ChIP-seq for SMARCA4 was performed in HCT116 cells and examined motif analysis. AP-1 family members were most enriched in the SMARCA4 bound regions (Figure 4E), suggesting that the SWI/SNF complex modulates chromatin state at the AP-1 family transcription factors bound site and regulates gene expression.

Fig. 4


A
HCT116
SNU484
HCT116 –SM4
SNU484 + SM4
H522
Input
IP: IgG
IP: SMCB1
SWI/SNF
INO80
SMARCA4
ACTL6A
SS18
SMARCC1
SMARCB1
INO80
YY1
SS18
SS18 / IgG
IgG
SMARCB1
B
HCT116
SNU484
HCT116 –SM4
SNU484 + SM4
H522
Input
IP: IgG
IP: SMCC1
SWI/SNF
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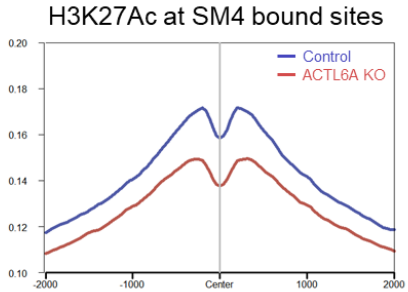
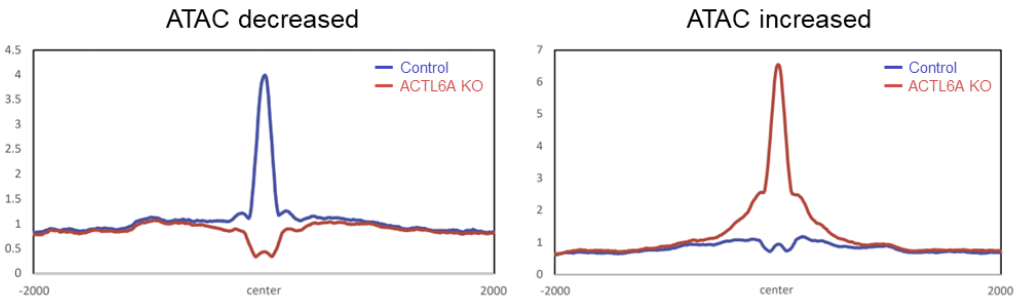
YY1
SS18
SS18 / IgG

C



D



E

Top known motif enrichments

Rank	motif	*p*-value
1	Fosl2	1e-8310
2	Fra2	1e-8294
3	Fra1	1e-8185
4	Jun-AP1	1e-8087
5	JunB	1e-8030
6	Fos	1e-7959
7	Atf3	1e-7599
8	BATF	1e-7491
9	AP-1	1e-6859

Figure 4. ACTL6A is required for SWI/SNF-mediated chromatin organization

(A) Co-immunoprecipitation (Co-IP) experiments using SMARCB1 antibody. Shown are western blots of SWI/SNF and INO80 subunits co-immunoprecipitated by SMARCB1 in HCT116, SNU484, SMARCA4-depleted HCT116, SMARCA4-expressing SNU484, H522 cells. Arrows indicate SS18, SS18/IgG, IgG and SMARCB1 accordingly.

(B) Co-immunoprecipitation (Co-IP) experiments using SMARCC1 antibody. Shown are western blots of SWI/SNF and INO80 subunits co-immunoprecipitated by SMARCC1 in HCT116, SNU484, SMARCA4-depleted HCT116, SMARCA4-expressing SNU484, H522 cells. Arrows indicate SS18, SS18/IgG accordingly.

(C) Metagene profiles of H3K27ac density at SMARCA4 bound sites in control (blue) and ACTL6A KO (red) cells.

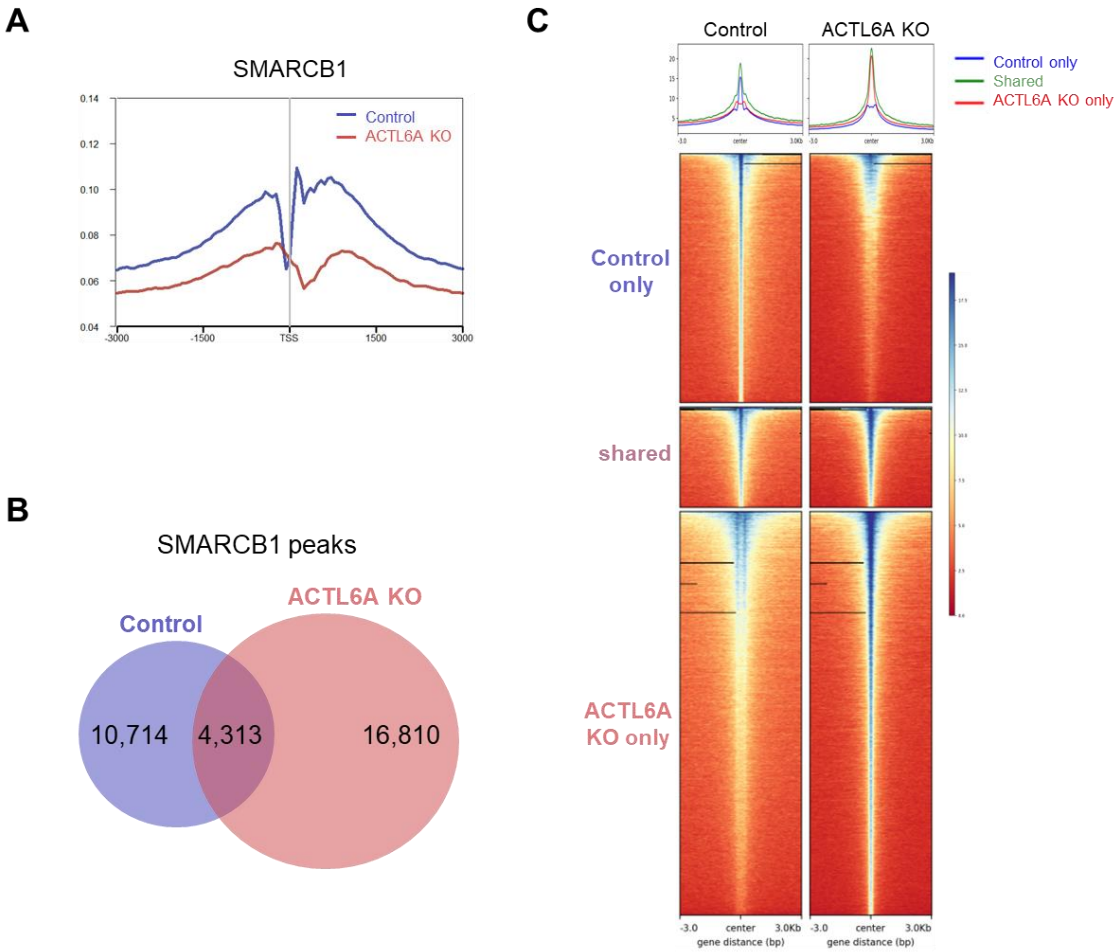
(D) Metagene profiles of ATAC-seq in control (blue) and ACTL6A depletion (red) HCT116 cells at SMARCA4 bound regions. ATAC decreased (n=5,137) and increased (n=4,088) deregulated regions.

(E) Top known motifs for SMARCA4 bound regions in HCT116 cells identified by HOMER.

ACTL6A deficiency leads to altered genomic localization of SWI/SNF complex

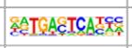
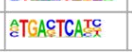
Since ATPase subunit SMARCA4 is destabilized as ACTL6A reduces (Figure 3B), ChIP-seq for SMARCB1 was performed to specifically examine the function of ACTL6A relate to SWI/SNF complex. SMARCB1 occupancy was decreased at the transcription start site (TSS) ±3kb in ACTL6A-depleted HCT116 cells compared to control cells (Figure 5A). Interestingly, a significant genome-wide increase in SMARCB1 enrichment (1.5-fold) was observed upon ACTL6A knockout (Figure 5B). Next, "control only", "shared", "ACTL6A KO only" genomic regions were identified, showing SMARCB1 binding in ACTL6A knockout cells compared to control cells. It was observed that SMARCB1 signal decreased in the "control only" region, whereas increased in the "ACTL6A KO only" region (Figure 5C). Motif analysis was performed in each of the regions. AP-1 motifs were enriched in all three regions (Figure 5D). This suggests that the ACTL6A-deficient SWI/SNF remain present in the absence of ACTL6A and may localize to a different AP-1 motifs than the AP-1 motifs to which the wild-type SWI/SNF complex binds. It is speculated that 4,313 "shared" regions are primarily unaffected by ACTL6A loss, which is not compensated for by the ACTL6A related SWI/SNF complex. Collectively, loss of ACTL6A results in redistribution of the SWI/SNF complex and may change chromatin structure.

Fig. 5



D

Control only

Rank	name	motif	*p*-value
1	Fosl2		1e-105
3	Jun-AP1		1e-101
4	JunB		1e-98
9	AP-1		1e-81

shared

Rank	name	motif	*p*-value
1	Fosl2		1e-1691
3	Fos		1e-1683
5	Jun-AP1		1e-1676
9	AP-1		1e-1487

ACTL6A KO only

Rank	name	motif	*p*-value
1	Fos		1e-8002
4	JunB		1e-7729
7	Fosl2		1e-7362
8	AP-1		1e-7248

Figure 5. ACTL6A deficiency leads to altered genomic localization of SWI/SNF complex

(A) Metagene plot of SMARCB1 signal at ±3kb TSS at SMARCB1 bound sites in control (blue) and ACTL6A KO (red) cells.

(B) Venn diagram showing SMARCB1 ChIP-seq peaks of control and ACTL6A knockout HCT116 cells.

(C) Heatmaps of ChIP-seq enrichment for SMARCB1 in control and ACTL6A-depletion HCT116 cells were centered on control only, shared, ACTL6A KO only regions.

(D) Motif analysis of the control only, shared, and ACTL6A KO only regions.

ACTL6A loss results in decreased genome wide INO80 occupancy and altered chromatin organization

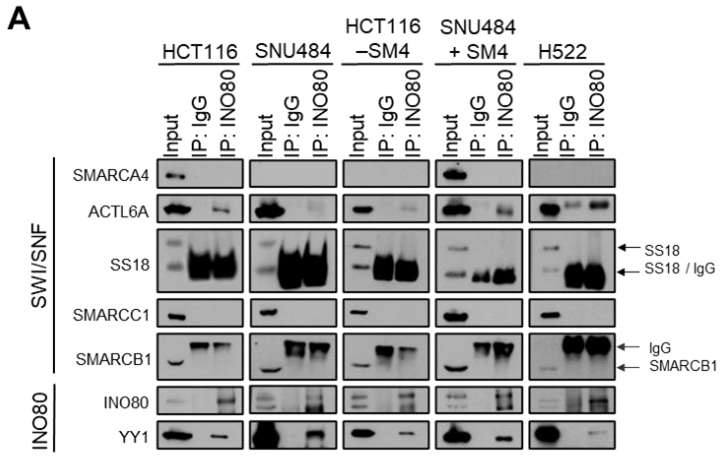
As shown in previous results, the loss of ACTL6A in SMARCA4/2^{Def} cells resulted in decreased cell proliferation (Figure 3B). In addition, ACTL6A loss in re-expressing SNU484 cells showed reduced cell growth (Figure 3D). So, I hypothesized that another chromatin remodeler, INO80 complex, may regulate chromatin state and cell proliferation independent of SWI/SNF complex. To investigate endogenous interaction between ACTL6A and INO80 complex, co-immunoprecipitation was performed. Co-IP of INO80 showed binding with ACTL6A regardless of SMARCA4/2. YY1 was also recovered by immunoprecipitation with antibody to INO80, consistent with prior reports that YY1 can associate with INO80 complex for transcriptional activation and DNA repair [15, 17]. SMARCA4, SS18, SMARCB1 and SMARCC1 were not detected in the INO80 complex in SMARCA4/2^{Pro} HCT116 cells. The same immunoprecipitations carried out in SMARCA4/2^{Def} SNU484 cells again clearly showed an association of ACTL6A and INO80. Immunoprecipitations with INO80 antibody in SMARCA4-depleted HCT116 cells also demonstrated that SMARCA4 do not co-precipitate ACTL6A and INO80. Furthermore, SMARCA4 re-expression in SMARCA4/2^{Def} SNU484 cells had no obvious effect on incorporation of ACTL6A with INO80, suggesting that INO80 complex is independent of SWI/SNF complex (Figure 6A).

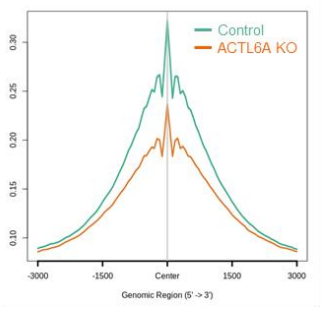
To further assess the impact of ACTL6A loss on INO80 complex, my ChIP-seq data for H3K27ac and ATAC-data was utilized, and

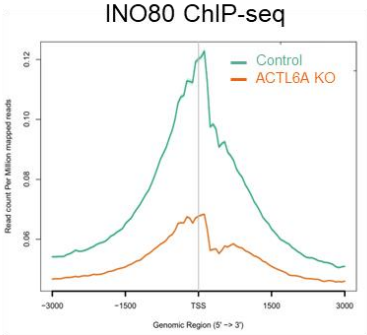
performed ChIP-seq for INO80 in ACTL6A-depleted HCT116 cells. Histone acetylation of INO80 bound sites was reduced in the ACTL6A knockout sample of HCT116 cells (Figure 6B). ACTL6A depletion induced loss of INO80 occupancy across the genome (Figure 6C). In addition, loss of ACTL6A resulted in altered ATAC-seq signals at INO80 bound regions (Figure 6D). Similar to motif analysis for SMARCA4 bound regions, Various AP-1 family members were also the most significantly enriched motif within INO80 bound sites (Figure 6E).

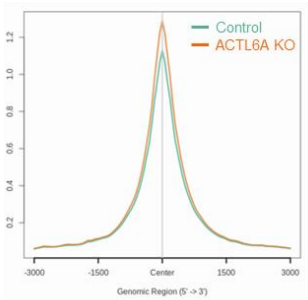
By intersecting the SMARCB1 ChIP-seq with the INO80 ChIP-seq, only 10% of peaks overlapped, which is consistent with previous results, suggesting that SWI/SNF and INO80 play independent roles (Figure 6F). 90% of INO80 enrichment was reduced genome wide in ACTL6A deficient cells compared to control cells, suggesting that ACTL6A plays a role in the binding of INO80 on the genome (Figure 6G). To investigate whether binding site of INO80 on genome was in fact altered in three regions (control only, shared, ACTL6A KO only), motif analysis was performed in each of the regions. AP-1 motifs were enriched in "control only" regions, while ETS1 and/or YY1 motifs were highly enriched in "shared" and "ACTL6A KO only" regions (Figure 6H). These findings highlight that the absence of ACTL6A reduces the occupancy of the INO80 complex that binds to the AP-1 motif, leading to changes in chromatin status and gene expression. Taken together, ACTL6A allows INO80 to bind to AP-1 motifs and regulates histone modification and chromatin accessibility.

Fig. 6







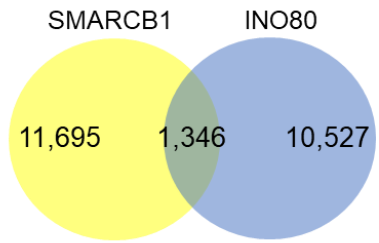


E

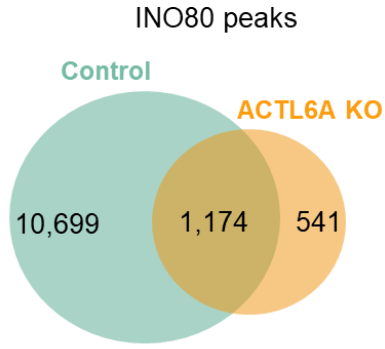
Top known motif enrichments

Rank	motif	*p*-value
1	YY1	1e-407
2	Jun-AP1	1e-390
3	Fosl2	1e-373
4	Fra1	1e-346
5	JunB	1e-340
6	Fos	1e-340
7	Fra2	1e-338
9	Atf3	1e-313
10	AP-1	1e-293

F



G



H

Control only

Rank	name	motif	*p*-value
1	Jun-AP1		1e-375
2	Fosl2		1e-370
5	JunB		1e-342
9	AP-1		1e-297
10	YY1		1e-270

shared

Rank	name	motif	*p*-value
1	YY1		1e-172
2	Elk4		1e-47
3	Fli1		1e-46
4	Elk1		1e-44

ACTL6A KO only

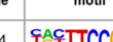
Rank	name	motif	*p*-value
1	Elk4		1e-17
2	ETS		1e-16
3	Elk1		1e-14
4	Elf1		1e-12

Figure 6. ACTL6A loss results in decreased genome-wide INO80 occupancy and altered chromatin organization

(A) Co-immunoprecipitation (Co-IP) experiments using INO80 antibody. Shown are western blots of SWI/SNF and INO80 subunits co-immunoprecipitated by INO80 in HCT116, SNU484, SMARCA4-depleted HCT116, SMARCA4-expressing SNU484, H522 cells.

(B) Metagene plot of H3K27ac density at INO80 bound sites in control (green) and ACTL6A depletion (orange) cells. The mean signal is determined by averaging signals of 3kb around the center of the indicated regions.

(C) Metagene plot of INO80 signal at ±3kb TSS in control (green) and ACTL6A KO (orange) HCT116 cells.

(D) Average ATAC-seq read density in control (green) and ACTL6A depletion (orange) HCT116 cells at INO80 bound regions (±3kb).

(E) Top known motifs for INO80 bound regions in HCT116 control cells identified by HOMER.

(F) Intersection of significant peaks between SMARCB1 and INO80 ChIP-seq in control HCT116 cells.

(G) Venn diagram showing INO80 ChIP-seq peaks of control and ACTL6A knockout HCT116 cells.

(H) Motif analysis of the control only, shared, and ACTL6A KO only regions.

SWI/SNF and INO80 complex regulates transcription on AP-1 binding sites

AP-1 (Activator protein-1) is a dimeric transcription factor that composed of AP-1 family members including Jun and Fos. AP-1 regulates cellular processes including cell proliferation, differentiation, survival and cell migration. AP-1 family members are well known to promote oncogenesis in various tumors, especially FOSL1 and JUN, which are highly expressed in invasive cancers and can mediate enhanced migration and proliferation [23, 24].

SWI/SNF and INO80 complex regulate the AP-1 binding site through ACTL6A (Figure 4E, 6E). Accordingly, I hypothesized that loss of ACTL6A affects transcription of the downstream genes as AP-1 does not work properly, and cell proliferation would be decreased. To verify the requirement of AP-1 proteins, main AP-1 family members, c-Fos and c-Jun, were knocked out in HCT116 cells. Fos and Jun were depleted respectively and were co-deleted to generate double knockout in HCT116 cells (Figure 7A). Cell proliferation was reduced, indicating that AP-1 factors are essential (Figure 7B). Collectively, results of AP-1 loss-of-function assay indicate that AP-1 factors are essential for SWI/SNF and INO80 complex transcription. The SWI/SNF and INO80 complex occupy the AP-1 motif, which in turn regulates gene expression as chromatin status changes.

I demonstrated the role of ACTL6A in relation to the SWI/SNF and INO80 complexes. ACTL6A loss leads to altered genomic

redistribution of SWI/SNF complex and reduction of INO80 enrichment. As a result, altered occupancy of these two complexes lead to changes in chromatin accessibility and histone modification, eventually to changes in gene expression, which affects the regulation of cell proliferation (Figure 7C and 7D).

Fig. 7

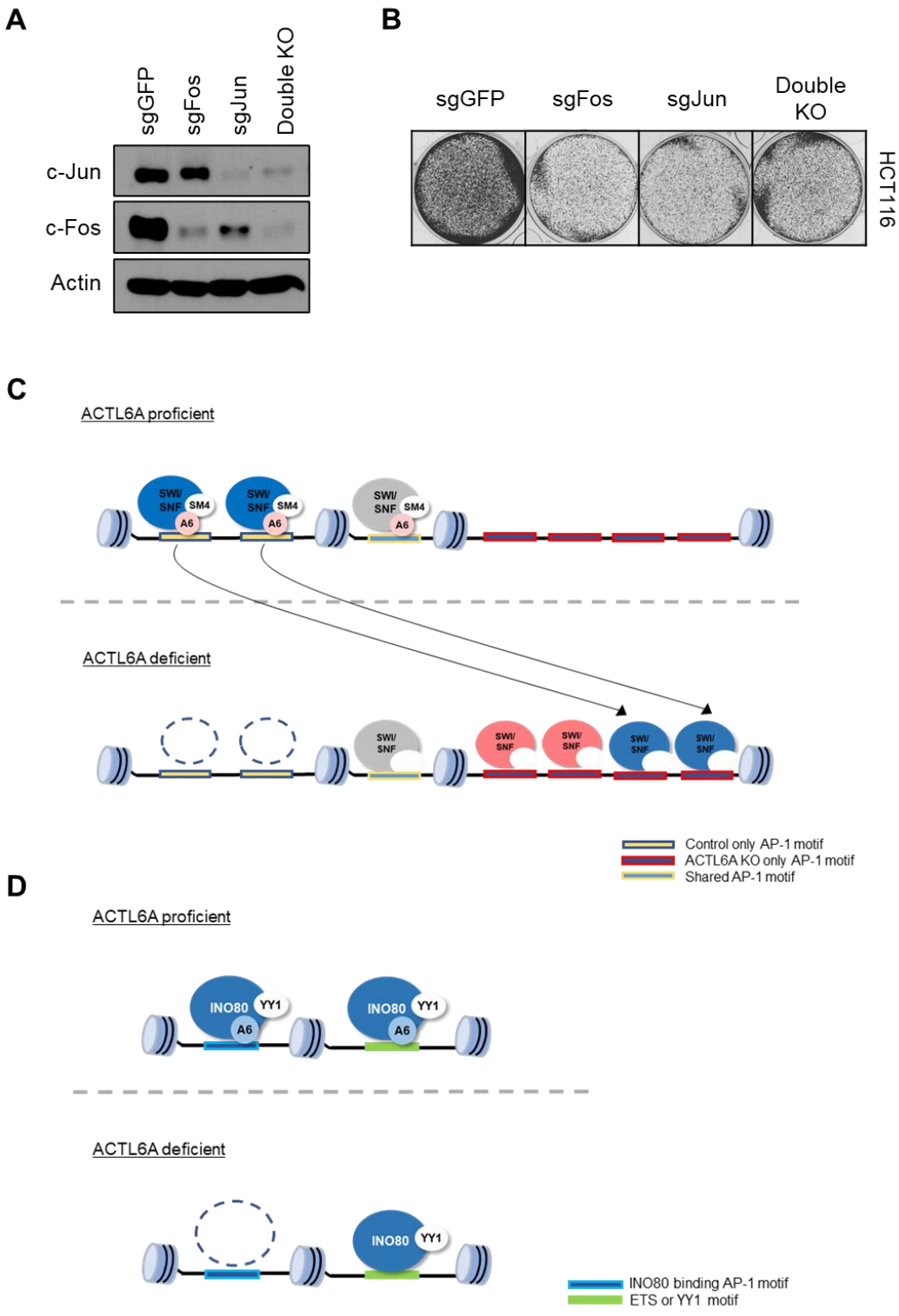

A
sgGFP
sgFos
sgJun
Double KO
c-Jun
c-Fos
Actin
B
sgGFP
sgFos
sgJun
Double KO
HCT116
C
ACTL6A proficient
SWI/SNF
SM4
A6
ACTL6A deficient
Control only AP-1 motif
ACTL6A KO only AP-1 motif
Shared AP-1 motif
D
ACTL6A proficient
INO80
YY1
A6
ACTL6A deficient
INO80 binding AP-1 motif
ETS or YY1 motif

Figure 7. SWI/SNF and INO80 complex regulates transcription on AP-1 binding sites

(A) Western blot analysis of HCT116 cells transduced with the indicated sgRNAs. Whole cell extracts were prepared on day 7 post transduction. Actin was used as a loading control.

(B) Colony forming assay after depletion of indicated sgRNAs in HCT116 cells.

(C), (D) Mechanism model of ACTL6A regulating chromatin dynamics.

Discussion

My study showed that ACTL6A is required for proper chromatin organization through interaction with the INO80 complex as well as the SWI/SNF complex. In the loss of function study, ACTL6A knockout is more effective in cell growth inhibition than SMARCA4/2 knockout in AGS and HCT116, suggesting that ACTL6A is essential for cell proliferation in gastrointestinal cells. Although SMARCA4 re-expression in SMARCA4/2-deficient SNU484 cell line showed increased cell growth, depletion of ACTL6A showed significant cell growth arrest. These results indicate that ACTL6A mediates cell proliferation regardless of SMARCA4/2 deficiency.

ACTL6A, as an epigenetic modifier, plays a role in transcriptional activation and repression of target genes by chromatin remodeling. ChIP-seq for histone active marker H3K27ac and ATAC-seq was performed. Loss of ACTL6A reduced H3K27ac levels in both promoter and enhancer, and changed genome-wide chromatin accessibility. GSEA of the DEGs of ACTL6A depletion cells showed enrichment in "p53 pathway", "apoptosis", and cell cycle-related pathways as well as "histone modification", "chromatin remodeling", and "ATP-dependent activity acting on DNA", indicating that ACTL6A has an essential role in chromatin remodeling via ATP-dependent chromatin remodeling complexes.

By co-immunoprecipitation with antibody SMARCB1, a core subunit of SWI/SNF complex, in HCT116 cells, the interaction of SMARCB1

with ACTL6A and other SWI/SNF complexes including SMARCA4, SMARCC1, SS18 was observed. Co-IP in SMARCA4-depleted HCT116 cells, SMARCB1 did not interact with ACTL6A, SMARCA4 and SS18, but still interacted with SMARCC1. In the SNU484 cell line, there was no interaction between SMARCB1 with ACTL6A and other SWI/SNF subunits except SMARCC1. In SMARCA4-expressing SNU484 cells, ACTL6A, SMARCA4, SS18 was recovered. My results demonstrate that SMARCA4 is required for ACTL6A to interact with SWI/SNF complex. Also, Co-IP with antibody INO80 showed an interaction with ACTL6A and YY1, but not with SWI/SNF subunits in all three SMARCA4/2 conditions, indicating that the SWI/SNF and INO80 complexes interacts with ACTL6A independently.

Interestingly, SMARCB1 ChIP-seq data showed that SWI/SNF complex enrichment was increased in ACTL6A-depleted HCT116 cells. Also, AP-1 transcription factor family members were most enriched in control only, shared, ACTL6A KO only regions, respectively. These results suggest that upon the loss of ACTL6A, the ACTL6A-deficient SWI/SNF complex binds to an AP-1 motif other than the AP-1 motif to which WT SWI/SNF was originally bound, resulting in redistribution. INO80 ChIP-seq in ACTL6A-depleted HCT116 cells was conducted, and INO80 enrichment was significantly reduced. Also, AP-1 family members were enriched in control only regions, while ETS and/or YY1 motif was enriched in shared and ACTL6A KO only region. This indicates that the loss of ACTL6A reduces the occupancy of the INO80 complex that binds to

the AP-1 motif, leading to chromatin status alteration and changed gene expressions.

Through the motif analysis results, it was confirmed that the SWI/SNF and INO80 complex binds to the AP-1 family members motif. Loss of ACTL6A changes SWI/SNF and INO80 complex occupancy, leading to changes in AP-1 target gene expression. To this end, c-Fos and c-Jun (representative AP-1 TFs) were knocked out, and cell proliferation was reduced.

My findings revealed that two chromatin remodelers SWI/SNF and INO80 complex are independently regulated through ACTL6A. And ACTL6A depletion resulted in SWI/SNF complex redistribution and reduced INO80 complex enrichment at the AP-1 motif. This may lead to changes in cell proliferation and survival-related genes. But further investigation will be needed to validate motif predictions, performing ChIP-seq for c-Fos or c-Jun. Finally, ACTL6A overexpression may play a role in the dynamics of SWI/SNF and INO80 complex occupancy in gastrointestinal cancer, suggesting ACTL6A as a therapeutic target.

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국문 초록

크로마틴 리모델링 복합체는 ATP 촉매를 통해 게놈 상의 크로마틴 접근성 및 구조를 조절하여 표적 유전자 발현을 제어할 수 있다. 그동안 SWI/SNF 복합체 서브유닛의 분자 메커니즘이 활발히 연구되어왔는데, 그 이유는 암의 25%가 SWI/SNF 서브유닛의 돌연변이로 인해 발생하기 때문이다. 다른 ATP 의존 크로마틴 리모델링 복합체로는 INO80 복합체가 있으며 전사 및 DNA 복구의 기능을 한다. INO80 서브유닛들은 대장암에서 높은 증폭률을 가지며 단백질 발현이 높다고 알려져 있다. ACTL6A 는 SWI/SNF 복합체와 INO80 복합체의 서브유닛 중 하나이다.

이전의 연구에서 CRISPR/Cas9 스크리닝을 통해 위암의 세포 성장에 역할을 하는 후성유전학적 변형인자인 ACTL6A 를 발견하였다. ACTL6A 의 단백질 및 mRNA 발현 수준은 정상의 위 및 대장 세포주보다 위암 및 대장암 세포주에서 더 높음을 확인했다. 하지만 위장관암의 증식에 관여하는 ACTL6A 의 메커니즘에 대해서는 아직 정확하게 보고된 바 없다. 따라서, 본 연구는 위장관암 세포에서 SWI/SNF 및 INO80 복합체와 관련한 ACTL6A 의 분자적 기전을 규명하고자 한다.

ACTL6A 는 SWI/SNF 복합체 및 INO80 복합체와 독립적으로 상호작용한다. 흥미롭게도, ACTL6A 고갈 시 SMARCB1 점유율이 게놈 전체에서 증가했으며, ACTL6A 억제 시 INO80 의 점유가 감소하는 것을 확인했다. 또한 ACTL6A 고갈 세포에서 SMARCB1 과 INO80 ChIP-seq 를 통한 모티프 분석 결과, AP-1 전사 인자 모티프들이 높게 나타났다.

이러한 결과들을 통해 ACTL6A 가 후성유전학적 변형인자로써 INO80 및 SWI/SNF 복합체와의 상호 작용을 통해 적절한 크로마틴 구성에 필요하다는 것을 확인했다. ACTL6A 억제 시 AP-1 모티프에서 두 복합체의 결합이 바뀌게 되면서 히스톤 변형 및 크로마틴 접근성이 변화하고, 이는 결국 세포 증식 조절에 관여하는 유전자들의 발현으로 이어진다는 것을 보여주었다. 본 연구를 통해서 ACTL6A 는 발암성 전사와 종양 형성을 촉진하는 데 중요한 역할을 한다는 것을 증명했으며 SWI/SNF 및 INO80 크로마틴 리모델링 복합체의 서브유닛인 ACTL6A 를 잠재적인 치료의 표적으로 제시하고자 한다.

주요어: ACTL6A; SWI/SNF 복합체; INO80 복합체; AP-1; 히스톤 변형; 크로마틴 리모델링; 위장관암; 후성유전학; 전사 조절

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