MLL5, a histone modifying enzyme, regulates androgen receptor activity in prostate cancer cells by recruiting co-regulators, HCF1 and SET1

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SUPPLEMENTARY MATERIALS AND METHODS

RNA isolation and RT-qPCR

Total cellular RNA was extracted using the Trizol reagent (Ambion, Austin, TX), according to the manufacturer’s instructions. For the induction of AR activity, 10 nM DHT was added after one day of serum deprivation. For each reverse-transcription reaction, 1 µg of total RNA was used for cDNA synthesis using the MultiScribe Reverse Transcription Kit from Life Technologies (Carlsbad, CA). Real-time PCR was performed using the EvaGreen qPCR Master Mix Kit from Applied Biological Materials Inc. (Richmond, BC, Canada) and a StepOne™ Real-Time PCR System (Applied Biosystems, CA). The quantity of 18S ribosomal RNA was measured as an internal control. Primers use are listed in Supplemental table S1.

Western blotting and co-immunoprecipitation

Cells (5x10⁶) and ground tissue (50–200 mg) were lysed in 1ml RIPA buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.2], 0.5% NP-40, 1% Triton X-100, and 1% sodium deoxycholate) containing a protease inhibitor cocktail (Sigma–Aldrich). Cell lysates were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore, Darmstadt, Germany). Membranes were blocked with 5% skim milk and 0.1% Tween-20 for 1 h and then incubated overnight at 4°C with the indicated primary antibodies. Membranes were incubated with a horseradish peroxidase–conjugated secondary antibody (1:5000) for 1 hour and developed using the ECL–Plus Kit (Thermo Scientific, Rockford, IL). For co-immunoprecipitation, 5x10⁶ LNCaP cells were kept serum free 24 h and treated with 10 nM DHT for another 24 h. Cell lysates (1 mg protein in RIPA buffer) were incubated with 1 µg of indicated antibody for 16 h and further incubated with 20 µl of protein A/G agarose beads (BE Healthcare Bio–Sciences, Piscataway, NJ) for 4 h. After washing with the excess volume
of wash buffer, the precipitated immune complexes were eluted in a denaturing SDS sample buffer, and then subjected to immunoblotting with the indicated antibodies.

**Cell migration assay and invasion assay**

Wound healing assay were performed on 100% confluent cells plated into 6-well culture plates. Straight scratches were made by using a pipette tip. Debris were removed by washing the cells twice, and fresh medium were added. Cells were incubated in a 5% CO₂ environment at 37°C, and observed using a SZX7 stereo microscope (Olympus, Tokyo, Japan) at the indicated time. For the invasion assay, cells (5 × 10^4/well) were plated in the upper chambers of Transwells without serum using Matrigel-coated polycarbonate membranes (Corning, Big Flats, NY). Basal medium containing 10% fetal bovine serum was added into the lower chambers as a chemoattractant for cell migration. After 48 h, non-migrated cells were removed from the upper chambers, while cells that migrated through chambers were fixed using 10% ethanol (Sigma–Aldrich). After cells were stained with the 0.01% crystal violet solution (Sigma–Aldrich), migrated cells were randomly counted in five different microscopic fields at 20 x magnification.

**Cell viability assay and colony formation assay**

For cell viability assay, cells (2,000 cells/well) were dispensed in 100 µl culture medium in a 96-well plate and incubated for the indicated time in a humidified incubator at 37°C with 5% CO₂. To perform cell viability analysis, EZ-Cytox cell viability kit (Daeil-Lab, Korea) solution (10 µl) was mixed with the culture medium in each well. The plate was incubated for 1 h at 37°C and absorbance was measured at 450 nm using a microplate reader (PerkinElmer, Waltham, MA). For colony formation assay, 1000 cells were plated in 6-well plates, cultured 14 days, and stained with 0.1% crystal violet. The colonies were photographed, and the number of colonies comprising more than 50 individual cells was counted using an SZX7 stereo microscope.
Chromatin Immunoprecipitation (ChIP) assays

ChIP was performed as described previously (13). Briefly, $1 \times 10^7$ cultured cells expressing indicated shRNA were serum-starved for 24 h and treated with 10 nM DHT for additional 24h.

Subsequently, the cells were cross-linked with 1% formaldehyde for 10 minutes, after which glycine (final concentration, 125 mM) was added to quench the cross-linking reaction. Cell lysates were subjected to sonication until the DNA was broken down to fragments of $< 500$ base pairs in size. Chromatin complexes were precipitated with control IgG or the indicated antibodies overnight at 4°C. Protein A/G Sepharose beads were added and the mixture incubated for 2 h. After washing the beads with saline, DNA–protein complexes were eluted with an elution buffer (1% SDS, 0.1 M NaHCO$_3$) and incubated overnight at 65°C to reverse the cross-links. The eluted DNA fragments were purified using the MEGA quick-spin™ DNA Purification Kit (Intron, Seoul, Korea). The precipitated DNAs were amplified and quantified employing the StepOne™ Real-Time PCR System using EvaGreen qPCR Master Mix. The primers used are listed in Supplemental table S1. All signals were normalized to input chromatin signals.

Animal studies

All animal experiments were performed in accordance with the institutional guidelines of Seoul National University Hospital under the IACUC protocol No.18-0189-S1A1. NOD scid gamma (NSG) mice were bred and maintained under Specific-pathogen-free conditions. Six-week-old male NSG mice were subcutaneously injected into the right flank with $1 \times 10^7$ human 22Rv1 cells expressing the indicated shRNA. To prepare the injections, cells were suspended in 100 µl of 50 % Matrigel (BD, NJ) in complete medium. Tumor growth was monitored externally.
using Vernier calipers for up to 40 days after the cell injection. The tumor volume was calculated as follows: tumor volume (mm$^3$) = length × (width)$^2$ × 0.5. At necropsy, the tumors were dissected and weighed. For all tumors, one portion was fixed in neutral buffered formalin for immunohistochemistry and the remaining piece was frozen and stored at -80°C for RNA and protein extraction.

**Human ethics approval and collection of human tissues**

The frozen tissues from prostate cancer patients were collected from Seoul Nation University Hospital Tissue Bank under approval of Institutional Review Board No. H-1607-135-777. The demographic data of each patient are shown in Supplemental Table S2. Normal and tumor tissues were identified from the pathology results. For western blotting, 50–200 mg of tissues was ground in liquid nitrogen and lysed with RIPA buffer.

**Statistical analyses and Oncomine analysis.**

All data were analyzed using Microsoft Excel 2016 software, unless otherwise stated. Continuous variables were analyzed using Student’s t-test if the data were normally distributed. All statistical tests were two-sided. Differences were considered significant in cases where p values were < 0.05. The expression levels of MLL5 gene in prostate cancer were analyzed using Oncomine database (www.oncomine.org). For this, we compared clinical specimens of cancer vs. normal patient datasets. We analyzed the results for their p-values, fold change.
Supplemental Fig. S1. *MLL5* mRNA is upregulated in prostate cancer patients. (A) *MLL5* mRNA expression levels in normal and prostate cancer tissues, based on the data obtained from the Oncomine database, are presented as box plots. N, normal prostate (n = 19 for Tomlins prostate, n = 8 for Vanaja prostate); T, prostate adenocarcinoma (n = 29 for Tomlins prostate, n = 27 for Vanaja prostate). (B) *MLL5* mRNA expression level in the indicated GSE datasets were evaluated. N, normal prostate (n = 6 for GSE 3325, n = 151 for GSE6919, n = 29 for GSE35988); L, localized prostate tumor (n = 7 for GSE 3325, n = 130 for GSE6919, n = 59 for GSE35988); M, metastatic prostate tumor (n = 6 for GSE 3325, n = 50 for GSE6919, n = 35 for GSE35988). Bars represent means ± SD of each group. * p < 0.05 (Student’s t-test) versus normal group.
Supplemental Fig. S2. MLL5 protein is upregulated in prostate cancer patients. (A) Proteins from prostate cancer tissues (T) and adjacent normal tissues (N) were analyzed through western blotting using the indicated antibodies. (B) Representative images of microarray data corresponding to MLL5 expression levels in prostate tumor and normal tissues. MLL5 expression levels in 73 different prostate tumor and 7 normal tissues were evaluated and plotted (right graph). Bars represent means ± SD of each group. *P < 0.05 (Student’s t-test) versus normal group.
Supplemental Fig. S3. Protein and mRNA levels of MLL5 in the indicated prostate cancer cells. (A) Whole cell extracts of each cell line were assessed using the indicated antibodies through western blotting. (B) The mRNA expression level of MLL5 was quantified in the indicated prostate cancer cells. Bars represent means ± SD of three independent experiments.
Supplemental Fig. S4. The effect of MLL5 knockdown on AR-negative prostate cancer cells. (A) The mRNA expression level of MLL5 was quantified in the indicated prostate cancer cells. Bars represent means ± SD of three independent experiments. * p < 0.05 (Student’s t-test) versus the control shRNA (sh-cont) group. (B) The rate of cell proliferation in PC3 and DU145 expressing MLL5 shRNAs. The time dependent viability changes of indicated cells were measured using EZ-Cytox solution. Relative O.D. was measured at the indicated time points. Bars represent means ± SD of three independent experiments.
Supplemental Fig. S5. MLL5 knock-down reduced cell mobility and EMT related gene expression in prostate cancer cell line LNCaP. (A) The protein level of indicated EMT markers was measured from whole cell extracts of control and MLL5 knockdown cells. Cells were treated with 10 nM DHT for 24 hours after one day of serum starvation. (B) The mRNA level of indicated EMT marker genes was measured from LNCaP cells with control or MLL5 shRNA expression. Cells were treated with 10 nM DHT for 24 hours after one day of serum starvation. (C) Scratch-wounding cell migration of the control and MLL5 shRNA expressing LNCaP cells were observed after indicated time. (D) The transwell infiltration assay of the same number of indicated stable cells. At 24 hours after plating, cells that had migrated to the underside of the filters were fixed and stained with crystal violet. Photographs were taken and relative cell migration was determined by the O.D 495 after extraction. Bars represents the means ± SDs of three independent experiments, and * denotes P < 0.05 (Student t-test) versus the sh-con group.
Supplemental Fig. S6

A

| Treatment  | + | - | + | - | + | - | + | - |
|------------|---|---|---|---|---|---|---|---|
| Sh_Con     |   |   |   |   |   |   |   |   |
| MLL5_sh01  |   |   |   |   | + |   |   |   |
| MLL5_sh02  |   |   |   |   |   | + |   |   |
| DHT (10nM) |   |   |   |   |   | + | + | + |

- α-H3K4me2
- α-H3K9me2
- α-H3K27me2
- α-Total Histone

B

- H3K4-Me2 % input
  - DHT (10nM)
  - Sh_con
  - Sh_MLL5_01
  - Sh_MLL5_02

- H3K9-Me2 % input
  - DHT (10nM)
  - Sh_con
  - Sh_MLL5_01
  - Sh_MLL5_02

KLK3
TMPRSS2
KLK2
Supplemental Fig. S6. Methylation status of AR target gene were changed in MLL5 knock-down prostate cancer cells. (A) LNCaP cells stably expressing control or MLL5 shRNA were treated with 10 nM DHT for 1 day, and cell extracts were immunoblotted with the indicated antibodies. (B) LNCaP cells with indicated shRNAs were treated with 10 nM DHT for 1 day, and Chromatin Immune-precipitation (ChIP) experiments were performed with the indicated antibodies (left). Immunoprecipitated DNA samples were subjected to qPCR with KLK3, TMPRSS2, and KLK2 gene promoter-sequence primers indicated on the bottom. Bars represents the means ± SDs of three independent experiments, and * denotes P < 0.05 (Student t-test) versus the sh-con group.
### Supplemental Table S1. Oligonucleotide sequences for RT–PCR and ChIP

| Primer sequences for Reverse transcription | Primer sequences for ChIP |
|-------------------------------------------|---------------------------|
| **18S_rRNA_RT_Fwd**                       | **hKLK3_ChIP_Fwd**        |
| **18S_rRNA_RT_Rev**                       | **GGAATCAGAGGAGTCTCACAA** |
| **hMLL5_RT_Fwd**                          | **hKLK3_ChIP_Rev**        |
| **hMLL5_RT_Rev**                          | **GCTAGCAGTGCCTGACATGC**  |
| **hKLK3_RT_Fwd**                          | **hKLK2_ChIP_Fwd**        |
| **hKLK3_RT_Rev**                          | **GCCTTCTGTGGCTTTGCC**    |
| **hTMPRSS2_RT_Fwd**                       | **hKLK2_ChIP_Rev**        |
| **hTMPRSS2_RT_Rev**                       | **GCACTTGCTTCCACACAT**    |
| **hIGF1R_RT_Fwd**                         | **hTMPRSS2_ChIP_Fwd**     |
| **hIGF1R_RT_Rev**                         | **TGTCCTGGATGATAAAAAAGTT**|
| **hKLK2_RT_Fwd**                          | **hTMPRSS2_ChIP_Rev**     |
| **hKLK2_RT_Rev**                          | **GACATACGCCCAACAAGAG**   |
| **hAR_RT_Fwd**                            |                            |
| **hAR_RT_Rev**                            |                            |
| **hCDH1_RT_Fwd**                          |                            |
| **hCDH1_RT_Rev**                          |                            |
| **hCDH2_RT_Fwd**                          |                            |
| **hCDH2_RT_Rev**                          |                            |
| **hVIM_RT_Fwd**                           |                            |
| **hVIM_RT_Rev**                           |                            |

Primer sequences for Reverse transcription:
- 18S_rRNA_RT_Fwd: TTCGTATTTGAGCCGCTAGA
- 18S_rRNA_RT_Rev: CTTTCGCTCTGGTCCGTCTT
- hMLL5_RT_Fwd: CCTCAACCCCACCAAGAGAC
- hMLL5_RT_Rev: AGACGTTTGGCTGTTG
- hKLK3_RT_Fwd: CACCTGCTCGGATCTGCTG
- hKLK3_RT_Rev: CCACCTCCGGTAAATGCACCA
- hTMPRSS2_RT_Fwd: GGACAGTGTGACCTCAAAGA
- hTMPRSS2_RT_Rev: TTGCTGCCCATGAAACTTCC
- hIGF1R_RT_Fwd: GGGCATCAGGATGGAGAAA
- hIGF1R_RT_Rev: CACAGGCGTTGCGTTG
- hKLK2_RT_Fwd: GCTGCCATTTGCTAAAGAG
- hKLK2_RT_Rev: TGGAAGCTGTCGCTGACA
- hAR_RT_Fwd: GGCAGACAGAGGGAAAGGG
- hAR_RT_Rev: CTTTGGTTCCTCCAGTCTT
- hCDH1_RT_Fwd: TCGGAACAGGACAAGTACC
- hCDH1_RT_Rev: ATCTTCACCTGCCGTCAGT
- hCDH2_RT_Fwd: GACATGCCCCTCAAGTGT
- hCDH2_RT_Rev: CCATTAAGCGAGTGGGT
- hVIM_RT_Fwd: GAGAACCTTTGGCCTGAAGC
- hVIM_RT_Rev: GCTTTCTGTAGGTGCAATC

Primer sequences for ChIP:
- hKLK3_ChIP_Fwd: GGGATCAGAGGAGTCTCACAA
- hKLK3_ChIP_Rev: GCTAGCAGTGCCTGACATGC
- hKLK2_ChIP_Fwd: GCCTTCTGCGTGTTCGCC
- hKLK2_ChIP_Rev: GCACTTGCTTCCACACAT
- hTMPRSS2_ChIP_Fwd: TGTCCTGGATGATAAAAAAGTTTT
- hTMPRSS2_ChIP_Rev: GACATACGCCCAACAAGAG
|   |   | age | iPSA  | T stage | pGS |
|---|---|-----|-------|---------|-----|
| 1 |   | 72  | 61    | 2c      | 7(4+3) |
| 2 |   | 77  | 9.73  | 2c      | 6(3+3) |
| 3 |   | 77  | 9.48  | 2c      | 6(3+3) |
| 4 |   | 62  | 7.25  | 2a      | 7(3+4) |
| 5 |   | 74  | 3.2   | 2c      | 7(3+4) |
| 6 |   | 65  | 8.07  | 2c      | 7(3+4) |
| 7 |   | 70  | 31.7  | 3a      | 8(4+4) |
| 8 |   | 62  | 13.9  | 3b      | 9(4+5) |