Supplementary Information of

NFκB Induces Epigenetic Repression of GPRC5A in Lung Epithelial Cells to Promote Neoplasia
Supplementary Data

Supplementary Figure 1 Related to Figure 4

Proteasome and autophagy inhibitors do not affect the repression of GPRC5A by TNFα and p65. **A**, Calu-1 cells were treated with TNFα (10 ng/ml) for 3 days and MG132 (10 μM) or chloroquine (CQ) (100 μM) were added to the medium for the last 6 hours, cells were lysed by RIPA. GPRC5A protein levels were analyzed by western blotting. **B**, Calu-1 cells expressed myc-p65 under the control of doxycycline (doxy)-inducible promoter (TRE2) were treated with doxycycline (100 ng/ml) to induce p65 expression for 48h. MG132 (10 μM) or chloroquine (100 μM) were added to the medium for the last 6 hours, cells were lysed by RIPA and GPRC5A protein levels were analyzed by western blotting.
Supplementary Figure 2 Related to Figure 7

DNA methylation at the GPRC5A promoter shows no different between lung cancer and adjacent normal tissues. A, three sequences (red color) in 2 CpG islands were chosen to perform DNA methylation sequencing. Sequence 1 and 2 were located in CpG island 1, includes 6 CpG sites respectively. Sequence 3 was located in CpG island 2, includes 5 CpG sites. All three sequences were indicated with red color. B, DNA were extract from ten lung cancer and corresponding adjacent normal tissues, DNA methylation levels on these three sequences were analyzed by bisulfite PCR; T, tumor; N, normal.
Overexpression of p65 in Calu-1 cells do not change the DNA methylation status at the GPRC5A promoter. Calu-1 cells expressed myc-p65 under the control of doxycycline-inducible promoter (TRE2) were treated with DMSO as negative control or doxycycline (300 ng/ml) to induce p65 expression for 24 h. The methylation status of the CpG island 1 (A) and CpG island 2 (B) at the GPRC5A promoter were analyzed by bisulfite sequencing PCR.

**Supplementary Figure 3 Related to Figure 7**
Supplementary Figure 4 Related to Figure 7

5-Aza-dc treatment has limited effects on the levels of RARβ and GPRC5A mRNA in three NSCLC cells. A549, H1975 and Calu-1 cells were treated with or without 5-Aza-dc (1 μM, 4 days). RARβ and GPRC5A mRNA levels were analyzed by quantitative PCR. *p<0.05; **p<0.01; ***p<0.001.
Supplementary Figure 5 Related to Figure 7

SAHA treatment increase GPRC5A mRNA levels. Normal human bronchial epithelial cell line (16HBE) and multiple human NSCLC cell lines were treated with or without SAHA (2.5 μM, 24 hours), GPRC5A mRNA levels were analyzed by quantitative PCR. *p<0.05; **p<0.01; ***p<0.001.
Supplementary Figure 6

SAHA treatment increases GPRC5A expression and inhibits tumor formation in vivo. A549 cells were injected subcutaneously into nude mice; mice were separated to two groups randomly. After three weeks, once group was treated with placebo and the other group was treated with SAHA. Tumor volumes were measured twice a week. After additional four weeks, mice were sacrificed. Tumor weights were measured, and GPRC5A mRNA and protein expression levels were analyzed by qPCR and western blot. *p<0.05; **p<0.01; ***p<0.001.
Supplementary Figure 7

NNK-treated Gprc5a-ko MTEC cells have stronger colony-formation capability than NNK-treated wild type METC cells in soft-agar assay. Wild type (WT) and Gprc5a-ko (KO) MTEC cells were repeatedly treated with NNK (100 pM) for 10 passages, leading to MTEC-KO-NNK10 and MTEC-WT-NNK10 cells. Five hundred cells per well (12 well plate) were seeded in soft agar with quadruplicates. After 3 weeks, the numbers and sizes of colonies were measured under microscope. *p<0.05; **p<0.01; ***p<0.001.
Supplementary Table 1. The primer sequence used in quantity real time PCR

| Gene   | Forward primer          | Reverse primer          |
|--------|-------------------------|-------------------------|
| mGprc5a | ACCACAGACTTTTGTGACCTGG  | CGAGTGCAAAACATGCAAGCC   |
| mAteb  | GGCTGTATTCCTCCATCG      | CCAGTTGGTAAACATGCCATGT |
| hGPRC5A| CTCACTCTCCCAGATCCTCGT   | CAGTCCGATGATGAAAGCCGAA |
| hRelA  | ATGTGGAGATCATTGACGCAC   | CCTGGTCTCTGTGAGCCATT    |
| hcIAP2 | TTTCCGTGGCTTTATTCAAACGT| GCACAGTGGTAGGAACCTTCAT  |
| hRARβ  | TCCGAAAAGCTCACCCAGGAA   | GGCAGTTCACTGAATTTGTC    |
| hGAPDH | TGCACCACCAACTGCTTAGGC   | GGCACTGGACTGTGGTCATGAG |
Supplementary Table 2. The primer sequence used in ChIP assay

| Gene    | Forward primer   | Reverse primer     |
|---------|------------------|--------------------|
| hGPRC5A | TGGAACTGGAATAGGC | GTGAACCTTGGTGC |
Uncropped/unedited gel document

Song et al (Submission to JCI Insight, August 2022)

**NF-κB Represses Retinoic Acid Receptor-Mediated Transactivation of GPRC5A in Lung Epithelial Cells for Neoplasia**

Note: This project was started from 2007 in Dr. Reuben Lotan’s laboratory at the University of Texas MD Anderson Cancer Center. It was carried out by Dr. Xiaofeng Ye (co-first author of this manuscript) as part of his PhD thesis under the direct supervision of Dr. Jiong Deng (research assistant professor at that time). Both of them were working on the GPRC5A project at Dr. Lotan’s group (please see reference below). Unfortunately, Dr. Lotan (1946-2011) passed away due to a terrible car accident in 2011, and his laboratory was closed 2-month later after this tragic accident. Dr. Ye graduated by summarizing his study/data on GPRC5A, including this uncompleted project, in his PhD thesis; he then performed a short period of post-doc study at Dr. Binhua P. Zhou’s laboratory working on cancer metastasis. Dr. Deng went back to Shanghai Jiao-Tong University School of Medicine (2010-2021) to continue the GPCR5A study, including this project in his laboratory. The first author (Dr. Hongyong Song) is a post-doctoral fellow at Dr. Deng’s group and he is the major driving force to finally complete this project. Due to the unexpected tragic accident of Dr. Lotan and the short notice of laboratory closure, Dr. Ye was unable to retrieve the original uncropped version of blots/films for Fig 2D, 3A, 3B, 5B, 6C and 6D despite many efforts.

To resolve these issues, we have repeated these experiments (for Fig 2D, 3A, 3B, 5B, 6C, and 6D) in the last three months. We have obtained similar results. Now we have presented these uncropped/unedited images in this uncropped/unedited blot documents.

**Reference:**

Xiaofeng Ye, Qingguo Tao, Yafan Wang, Yijun Cheng, **Reuben Lotan**. Mechanisms underlying the induction of the putative human tumor suppressor GPRC5A by retinoic acid. Cancer Biol Ther. 2009 May;8(10):951-62.

Qingguo Tao, Junya Fujimoto, Taoyan Men, *Xiaofeng Ye*, Jiong Deng, Ludovic Lacroix, John L Clifford, Li Mao, Carolyn S Van Pelt, J Jack Lee, Dafna Lotan, Reuben Lotan. Identification of the retinoic acid-inducible Gprc5a as a new lung tumor suppressor gene. J Natl Cancer Inst. 2007 Nov 21;99(22):1668-82.

Jiong Deng, Junya Fujimoto, *Xiao-Feng Ye*, Tao-Yan Men, Carolyn S Van Pelt, Yu-Long Chen, Xiao-Feng Lin, Humam Kadara, Qingguo Tao, Dafna Lotan, Reuben Lotan. Knockout of the tumor suppressor gene Gprc5a in mice leads to NF-kappaB activation in airway epithelium and promotes lung inflammation and tumorigenesis. Cancer Prev Res (Phila). 2010 Apr;3(4):424-37.
Small Airway Epithelial Cells

**Fig 2A**

| SAEC | C | ATRA | TNFα | CSC |
|------|---|------|------|-----|
|      |   |      |      |     |

GPRC5A

Actin

**Fig 2B**

**Original**

| Cell Line | TNFα |   |
|-----------|------|---|
| H460      | -    | + |
| A549      | -    | + |
| Calu-1    | -    | + |
| SK-Mos-1  | -    | + |
| 1299      | -    | + |
| 293G      | -    | + |
| H322      | -    | + |
| H358      | -    | + |

**IB: GPRC5A**

| Cell Line | TNFα |   |
|-----------|------|---|
| H460      | -    | + |
| A549      | -    | + |
| Calu-1    | -    | + |
| SK-Mos-1  | -    | + |
| 1299      | -    | + |
| 293G      | -    | + |
| H322      | -    | + |
| H358      | -    | + |

**β-actin**

**revised**

| Calu-1 | H292G | H322 |
|--------|-------|------|
| TNFα:  |       |      |
| -      | +     |      |
| -      | +     | -    |
| -      | +     | +    |
### Fig 2F

| LPS:  | 8  | 8  | 8  | 8  | 6  | 6  | 4  | 4  | 4  | 4  | -  | -  | -  | -  | Days |
|-------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|------|
| Gprc5a |    |    |    |    |    |    |    |    |    |    |    |    |    |    |      |
| Actin |    |    |    |    |    |    |    |    |    |    |    |    |    |    |      |

The figure shows a gel electrophoresis analysis with two panels: one for Gprc5a and another for Actin. The table indicates the LPS exposure times for WT and Gprc5a-ko groups over different days.
**Fig 3D**

The figure shows a protein blot analysis with Western blots for various proteins under different conditions. The blot images are labeled with the following proteins:

- GPRC5A
- p65
- β-actin

The blotting conditions include Doxy treatment and various protein constructs. The blot analysis compares the expression levels of these proteins under different treatment conditions, specifically highlighting the effects of Doxy on GPRC5A and p65. The blot images are arranged in a grid, with each row representing a different protein and each column representing a different experimental condition.

**Legend:**

- **IB:** Western blot analysis
- **Kd:** Kilodaltons
- **Doxy:** Doxycycline

The figure also includes a red box highlighting the blot images and a label indicating the original picture location.
Fig 3D
Fig 3D

GPRC5A and p65 blot were run in the same gel, loading the same volume of sample. And then were cropped to blot with different Ab. P65(1-364) were blotted with myc-tag Ab, and because p65(296-551) without any tag, so p65(296-551) were blotted with p65 Ab (target its c-terminal). The following picture was the original experimental record for this blot.
Fig 3E

**Table: myc-p65/ Calu-1**

|       | WT | S276A |
|-------|----|-------|
| Doxy: | -  | +     |

**Images:**
- **GPRC5A**
- **Myc-tag**
- **p-p65(S276)**
- **GAPDH**
Fig 4

|     | Calu-1 |
|-----|--------|
|     |        |
| TNFα | C  | 30' | 60' | CHX | 30' | 60' |

A

- **IκBα**
- **p-p65 (S536)**
- **p65**
- **β-actin**
Fig 4

D

| TNFα (h): | C  | 12 | 24 | CHX | 0  | 12 |
|-----------|----|----|----|-----|----|----|

- GPRC5A
- β-actin
### Table D

|        | Calu-1 |
|--------|--------|
| ATRA   | -      | +      | +      | +      |
| TNFα   | -      | +      | -      | +      |

### Figure D

**GPRC5A**

- Lane 1: ATRA-/-
- Lane 2: ATRA-/+ (Unintelligible)
- Lane 3: ATRA+/+
- Lane 4: ATRA++/

**p-p65(S536)**

- Lane 1: ATRA-/-
- Lane 2: ATRA-/+ (Unintelligible)
- Lane 3: ATRA+/+
- Lane 4: ATRA++/

**GAPDH**

- Lane 1: ATRA-/-
- Lane 2: ATRA-/+ (Unintelligible)
- Lane 3: ATRA+/+
- Lane 4: ATRA++/
Fig 6 E

|            | IP: Flag | N IgG |
|------------|----------|-------|
| Flag-RARα  | -        | +     |
| GFP        | -        | -     |
| GFP-p65-WT | -        | -     |
| GFP-p65-S276A | -     | +     |

IB: GFP

GFP-p65/-S276A

IB: Flag (RARα)

GFP

kD

55
70
35
130
100
70
55
25
Fig 7

GPRC5A

Tubulin

NSCLC cell lines

16HBE
PC9
H460
A549
Calu-1
HCC527
H1975
H1992
H1299

kD

-55
-40
-35

-55 kD
Fig 7

Calu-1

| Input | RNA pol II | H3K9ac | N IgG |
|-------|------------|--------|-------|
|       |            | M      |       |
| -     | +          | -      |       |
| -     | +          | -      |       |
| -     | +          | -      |       |
| +     | +          | -      |       |
| +     | +          | -      |       |
| N IgG| M          | -      |       |

B

GPRC5A

250bp

100bp

GAPDH

100bp

250bp

464bp

100bp
Fig 7

Calu-1 myc-p65

| WT | H3K9ac | N IgG |
|----|--------|-------|
| Doxy: | - | + | - | + |

| S276A | H3K9ac | N IgG |
|-------|--------|-------|
| Doxy: | - | + | - | + |

GPRC5A

GAPDH

164bp

166bp
Fig 7

|       | A549 |
|-------|------|
| 5-Aza-dc: | -    +  -  + |
| SAHA:    | -    -   +  + |

GPRC5A

GAPDH
Fig 7

F

5-Aza-dc: - + - +
SAHA: - - + +

GPRC5A

GAPDH
Fig 7

G

|          | Calu-1 |
|----------|--------|
| 5-Aza-dc:| -      |
| SAHA:    | -      |

GPRC5A

|          | Tubulin |
|----------|---------|
|          | 55 kD   |
| SAHA (h) | Input | RNAI | N IgG |
|---------|-------|------|-------|
| 0       | 24    | 0    | 24    |

**ChIP (Calu-1)**

**GPRC5A**

- 164bp
- 166bp

**GAPDH**

- 164bp
- 166bp
Fig 7

**J**

|          | 16HBE | H460 | H1299 | H1792 | HCC827 | PC9 |
|----------|-------|------|-------|-------|--------|-----|
| SAHA:    | -     | +    | -     | +     | -      | +   |

**GPRC5A**

**Tubulin**

55 kD
Fig 7

K

|          | Calu-1 |
|----------|--------|
| TNFα:    | DMSO   |
|          | 5-Aza-dc |
|          | SAHA   |
| -       | -      | -      | + |
| +       | -      | +      | - |
| -       | +      | -      | + |

- GPRC5A
- H3K9ac
- p-p65(S536)
- GAPDH

kD
- 70
- 55
- 40
- 35
- 20
Figure 2D

**Old data**

| Calu-1 | TNFα(h): 0 6 12 24 36 48 |
|--------|--------------------------|
| GPRC5A |
| HSP90  |

**New data**

| Calu-1 | TNFα(h): 0 6 12 24 36 48 |
|--------|--------------------------|
| GPRC5A |
| HSP90  |

Relative protein

TNFα(h): 0 6 12 24 36 48
Figure 3A

New data

old data
Figure 3B

New data

old data
Figure 5

Old data

New data

C

H157

| ATRA: | V | ASβ |
|-------|---|-----|
| ATRA: | - | +   |
| GPRC5A|   |     |
| β-actin|   |     |
| RARβ |   |     |

697bp
281bp
311bp

Calu1

sh-ns  sh-RARβ

ATRA: - + - +

GPRC5A

β-actin

RARβ

697bp
281bp
311bp
Figure 5B

Old data

New data
Figure 6C

Old data

New data

Old data → New data

Flag-RARAα+myc-p65
Flag-RARB+myc-p65

IB: myc
IB: Flag
Figure 6D

**Old data**

**New data**

| INPUT | IP: Flag | N IgG |
|-------|----------|-------|
| Flag-RARβ2 | + | - | + |
| Flag-RARβ4 | - | + | - |
| myc-p65 | + | + | + |

**IB:**
- Myc
- Flag

**Molecular Weight (kD):**
- 70
- 55
- 40
- 35
- 25
- 170
- 130
- 100
- 70
- 55
- 40
- 35
- 25