JCI insight

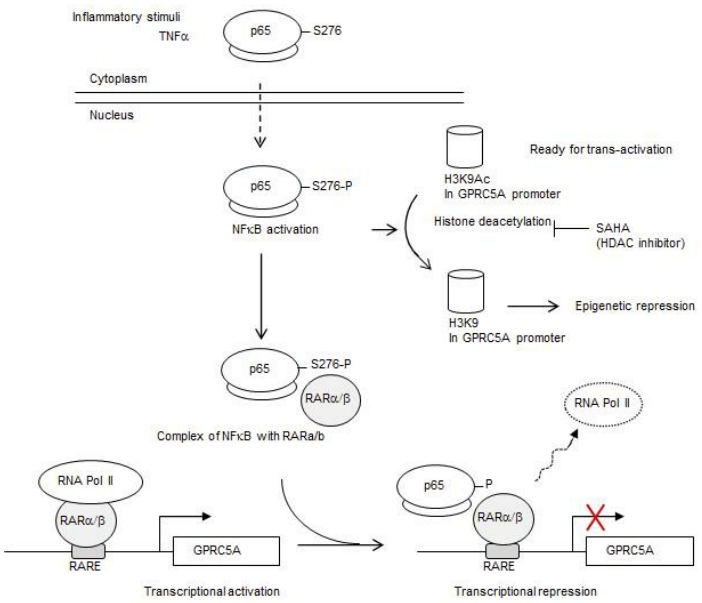
NFκB induces epigenetic repression of GPRC5A in lung epithelial cells to promote neoplasia

Hongyong Song, … , Binhua P. Zhou, Jiong Deng

JCI Insight. 2022. https://doi.org/10.1172/jci.insight.153976.

Research In-Press Preview **Oncology**

Graphical abstract



Find the latest version:

https://jci.me/153976/pdf

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

Hongyong Song[1,2‡], Xiaofeng Ye[3‡], Yueling Liao[1,4‡], Siwei Zhang[1,2], Dongliang Xu[1,2], Shuangshuang Zhong[1,2], Bo Jing[1,2], Tong Wang[1,2], Beibei Sun[1,2], Jianhua Xu[1,2], Wenzheng Guo[3], Kaimi Li[1,2], Min Hu[1,2], Yanbin Kuang[1,5], Jing Ling[1,2], Tuo Zhang[1,2], Yadi Wu[3], Jing Du[6], Feng Yao[1,2], Y Eugene Chin[6], Qi Wang[7], Binhua P. Zhou[3*], and Jiong Deng[1,2,6*]

[1]Key Laboratory of Cell Differentiation and Apoptosis of Chinese Minister of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, 200025 China

[2]Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine, Shanghai, 200025 China

[3]Department of Molecular and Cellular Biochemistry, Markey Cancer Center, University of Kentucky College of Medicine, Lexington, KY, USA

[4]Zhejiang Provincial Key Laboratory for Water Environment and Marine Biological Resources Protection, College of Life and Environmental Science, Wenzhou University, Wenzhou 325000, China

[5]Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, 200025 China

[6]Medical Research Center, Binzhou Medical University Hospital, Binzhou, Shandong 256600, China; and Peninsular Cancer Center, Binzhou Medical University, Yantai, Shandong 264003, China

[7]Department of Respiratory Medicine, the Second Affiliated Hospital, Dalian Medical University, Dalian, China

[‡]These authors contribute equally to this work

Conflict of interest statement:
The authors have declared the no conflict of interest exists.

[*]**Correspondence:**

Binhua P. Zhou, MD, PhD
University of Kentucky Markey Cancer Center
Email: peter.zhou@uky.edu

or

Jiong Deng, PhD
Binzhou Medical University Hospital
Email: jiongdeng@bzmc.edu.cn

Abstract

Chronic inflammation is associated with lung tumorigenesis, in which NF-κB-mediated epigenetic regulations play a critical role. Lung tumor suppressor GPRC5A is repressed in most non-small cell lung cancer (NSCLC), however the mechanisms remain unclear. Here, we show that NF-κB acts as a transcriptional repressor in suppression of GPRC5A. NF-κB induces GPRC5A repression both in vitro and in vivo. Intriguingly, trans-activation of NF-κB downstream targets is not required, but the trans-activation domain of RelA/p65 was required for GPRC5A repression. NF-κB did not bind to any potential cis-element in GPRC5A promoter. Instead, p65 was complexed with RARα/β, and recruited to the RA-response element (RARE) site at the GPRC5A promoter, resulting in disrupted RNA polymerase II complex, and suppressed transcription. Noticeably, phosphorylation on Serine276 of p65 is required for interaction with RARα/β and repression of GPRC5A. Moreover, NF-κB-mediated epigenetic repression is through suppression of histone H3K9ac, but not DNA methylation of the CpG islands, at the GPRC5A promoter. Consistently, a HDAC inhibitor, but not DNA methylation inhibitor, restored GPRC5A expression in NSCLC cells. Thus, NF-κB induces transcriptional repression of GPRC5A via complex with RARα/β and mediates epigenetic repression via suppression of H3K9ac.

Introduction

Tumorigenesis, a dedifferentiation process, is associated with chronic inflammation (1-3). However, the molecular mechanism that switches the expression profile from homeostasis to dedifferentiation remains elusive. Lung is an organ that expose to various environmental insults, such as bacteria and virus infection, air pollution or cigarette smoking. These risk factors trigger chronic inflammation which release reactive oxygen and nitrogen species (ROS and RNS), causing DNA damage and mutations (4). Pro-inflammatory cytokines from microenvironment can activate the NF-κB signaling pathway, a pivotal transcriptional factor for induction of multiple downstream genes, in target cells. These downstream NF-κB targets include molecules involved in anti-apoptosis, cell survival, proliferation, angiogenesis, and immune-evasion. Up to now, nearly all NF-κB-mediated functions are attributed to its trans-activation activities, or activation of NF-κB downstream genes. However, little is known about the role of NF-κB in repression of the genes for homeostasis or differentiation.

GPRC5A (G-protein-coupled receptor, family C, member 5A) was cloned as a retinoic acid-inducible gene 1 (RAIG1) (5). GPRC5A is predominately expressed in lung epithelial cells (6), suggesting that it plays a differentiation role in maintaining homeostasis of lung epithelium. *Gprc5a*-knockout (ko) mice have normal lung development, but developed spontaneous lung cancer in later life. Thus, Gprc5a is recognized as a lung tumor suppressor gene (6, 7). The biological functions of GPRC5A are linked to its roles in restraining EGFR signaling (8), inhibition of EGFR protein synthesis (9), inhibition of activated NF-κB and STAT3 signaling (10-12), and regulation of the MDM2-p53 pathway (13). Importantly, GPRC5A repression is found in human non-small cell lung cancer (NSCLC) samples and chronic obstructive pulmonary disease (COPD) tissues (6, 8, 14). Furthermore, GPRC5A repression was correlated with dedifferentiation grade of oral squamous cell carcinoma (OSCC) (15). These observations suggest that GPRC5A is a biomarker of differentiation in lung and oral epithelial cells, whereas repression of GPRC5A promotes dedifferentiation or pro-oncogenic process for neoplasia. However, the underlying mechanism of GPRC5A repression remains elusive.

In this study, we showed that NF-κB acts as a transcription repressor to repress GPRC5A expression. We showed that NF-κB, via interaction with retinoic acid receptor (RAR), inhibits RAR-mediated transcription of GPRC5A, switching the gene expression from homeostasis to dedifferentiation in lung epithelial cells for neoplasia.

Results

Active NF-κB is strongly correlated with GPRC5A repression in human NSCLC and COPD samples. GPRC5A is predominately expressed in lung epithelial cells, but its expression is often loss in lung cancer. To determine the relationship between NF-κB activation and GPRC5A expression, we measured the levels of GPRC5A expression and active p65 (nuclear p65) in samples via immunohistochemistry (IHC) staining. The results showed that GPRC5A expression was significantly repressed, whereas active p65 was greatly increased, in most of NSCLC, including squamous cell carcinoma (SCC) and adenocarcinoma (ADC), and all COPD tissues, compared to that in adjacent normal (AN) lung tissues (Figure 1A and B). Pearson correlation analysis showed that GPRC5A protein level and active p65 were inversely correlated in normal lung, NSCLC and COPD tissues (Figure 1C). Further analysis of TCGA database showed that the mRNA level of TNFα, a key cytokine responsible for NF-κB activation, was inversely correlated with GPRC5A expression in Lung adenocarcinoma (LUAD) (Figure 1D). Taken together, these results suggest that activated NF-κB is strongly correlated with GPRC5A repression.

Inflammatory stimuli repress GPRC5A expression both in vitro and in vivo. To determine the role of inflammatory signaling on GPRC5A expression, we examined the effects of TNFα in small airway epithelial cells (SAEC). Immunoblot assay showed that, all trans-retinoic acid (ATRA) induced GPRC5A expression, confirming that GPRC5A is a RA target gene (5, 16, 17) (Figure 2A). Interestingly, treatment with TNFα as well as cigarette smoking condense (CSC) suppressed ATRA-induced GPRC5A expression. Similarly, TNFα treatment inhibited GPRC5A expression in several NSCLC cell lines (Calu-1, H322 and H292G cells) (Figure 2B). Q-PCR analysis showed that the mRNA level of GPRC5A was significantly suppressed after 6 hours of TNFα treatment (Figure 2C). In comparison, GPRC5A protein level started to decrease after 12 hours of TNFα treatment in Calu-1 cells (Figure 2D). These results suggest that TNFα represses GPRC5A at transcriptional level.

Next, we examined the effect of inflammation on GPRC5A repression *in vivo* using an NF-κB-driven luciferase (NF-κB-luc) mouse model. After aero-exposure of these mice to LPS (aero-LPS) for 30 min, luciferin was injected intraperitoneally (i.p.) to visualize NF-κB activation. The images showed that aero-LPS exposure induced intensive bioluminescence in the lungs of these mice (Figure 2E, top panel), quantitation of relative bioluminescence intensity confirmed NF-κB activation in these mice (Figure 2E, bottom panel). When lung tissues from these mice were analyzed for Gprc5a expression, immunoblot showed that Gprc5a was significantly suppressed following aero-LPS exposure for 4-8 days (Figure 2F). Consistently, Q-PCR analysis showed that Gprc5a mRNA was greatly suppressed in mouse lung tissues treated with aero-LPS for 4 day compared to untreated ones

(Figure 2G), indicating that NF-κB-mediated repression of Gprc5a occurs at the transcription level. Taken together, inflammatory stimuli, TNFα used in vitro or LPS used in vivo, significantly suppress GPRC5A expression at the transcriptional level both *in vitro* and *in vivo*.

The trans-activation domain of p65 is essential for NF-κB-mediated GPRC5A repression. NF-κB is a major intracellular mediator of inflammatory signaling elicited from TNFα. To determine whether NF-κB is the mediator responsible for TNFα-induced GPRC5A repression, we examined the role of TNFα in cancer cells treated with either RelA/p65 (subunit of NF-κB) knockdown by small interfering RNA (siRNA) or overexpression of dominant negative inhibitor IκBα S32/36A mutant (IκBα-AA). The immunoblot assay showed that p65-knockdown abolished TNFα-induced GPRC5A repression in Calu-1 cells (Figure 3A). Similarly, over-expression of IκBα-AA blocked TNFα-induced GPRC5A repression (Figure 3B). This suggests that TNFα-induced repression of GPRC5A is through NF-κB.

To define the functional domain of p65 that is responsible for the repression, we constructed inducible lentiviral expression constructs of full length (FL) p65, or its mutant with deleted trans-activation domain (TAD, 1-440 residues) or Rel homology domain (RHD, 296-551 residues) (Figure 3C), and established stable clones in Calu-1 cells. Immunoblot assay showed that doxycycline (dox)-induced expression of p65 (FL) suppressed GPRC5A expression, whereas deletion mutants, including p65-(1-440) and p65-(296-551), did not (Figure 3D). These suggest that both RHD and TAD domains of p65 are required for GPRC5A repression.

Post-translation modifications, including phosphorylation, are known to play an important role in regulating the trans-activation activities of RelA/p65 (18-20). Previously, it was shown that phosphorylation of serine-276 is critical for p65-mediated repression of BRMS1 (breast cancer metastasis suppressor 1) (21). We asked if serine-276 is essential for repression of GPRC5A. To determine the role of S276 of p65, we established inducible clones, expressing either wild type (WT) p65 or p65-S276A mutant in Calu-1 cells. Immunoblot analysis showed that WT-p65 suppressed GPRC5A expression whereas p65-S276A did not (Figure 3E). Consistently, Q-PCR analysis showed that expression of WT-p65 repressed GPRC5A at mRNA level, whereas p65-S276A mutant did not (Figure 3F). Taken together, the S276 site in the RHD domain of p65 is critical for NF-κB-mediated repression of GPRC5A.

NF-κB-mediated GPRC5A repression is independent of its trans-activation functions. NF-κB is known to act as a transcription activator. One possibility is that NF-κB-mediated repression is through trans-activation of its downstream target genes, which indirectly repress GPRC5A expression. To test

this possibility, we examined the effects of protein translation inhibitor cycloheximide (CHX) on NF-κB-induced trans-activation and trans-repression. Immunoblot analysis showed that TNFα induced IκBα degradation at 30 minutes in Calu-1 cells (Figure 4A), via an ubiquitination pathway (22, 23); The IκBα level was restored at 60 minutes through de novo protein synthesis (Figure 4A) since IκBα is a well-known NF-κB target gene (22). Although CHX treatment blocked IκBα expression at 60 minutes (Figure 4A), it did not block the IκBα mRNA level (Figure 4B), indicating that CHX treatment indeed blocked newly protein synthesis. Importantly, CHX treatment, which blocks protein synthesis, did not alter TNFα-induced GPRC5A repression at both the mRNA (Figure 4C) and protein levels (Figure 4D). These suggest that trans-activation of NF-κB downstream target genes is not required for TNFα-induced GPRC5A repression. In addition, treatment with proteasome inhibitor MG132 and autophagy inhibitor chloroquine (CQ) had no effect on GPRC5A level following TNFα treatment or p65 induction (Supplementary Figure 1), supporting that NF-κB-mediated GPRC5A repression is regulated at the transcriptional level. Taken together, NF-κB represses GPRC5A expression at transcriptional level, which is independent of trans-activation of NF-κB downstream target genes.

Next, we asked whether NF-κB acts via direct binding to the potential NF-κB response cis-element (NFRE) in the GPRC5A promoter. By screening potential NFRE-like sequences in GPRC5A promoter via software, we found that there are three potential NFRE sites in the promoter region. To determine whether these sites, designated as A, B, C (Figure 4E, upper), play a role in NF-κB-mediated transcriptional GPRC5A repression, we examined the effect of p65 on GPRC5A promoter-driven luciferase reporters (GPRC5A-luc) with various mutations on these sites. Transfection of HEK293T cells with various constructs showed that p65 repressed the luciferase activity from GPRC5A-luc. Importantly, mutation of any of three potential sites or different combination did not affect p65-mediated repression of GPRC5A-luc reporters (Figure 4E, below). This suggests that, none of three potential NF-κB-binding cis-elements is required for NF-κB-mediated repression. Instead, we found that the construct GPRC5A-luc-DR5mut, which has mutated retinoic acid response element (RARE) in the promoter of GPRC5A (24), completely lost luciferase activity regardless of the expression of NF-κB p65 subunit (Figure 4F). This suggests that RARE at the GPRC5A promoter is critical for regulating its expression. Thus, it raises an interesting question if NF-κB-mediated repression of GPRC5A is through disrupting the transcription complex at RARE in its promoter.

NF-κB represses GPRC5A via inhibiting RA signaling. Since GPRC5A is a RA target gene, we asked whether NF-κB represses GPRC5A expression via inhibiting RA-mediated transcription. First, we examined the role of the RA signaling pathway in GPRC5A expression. All-trans retinoic acid (ATRA) treatment induced both GPRC5A and RARβ in NSCLC cell line H157 by RT-PCR analysis;

however, the induction was eliminated when RARβ is knockdown by siRNA (Figure 5A). These suggest that RARβ is essential for GPRC5A expression. Consistently, immunoblot analysis showed that, ATRA could induce GPRC5A in H157-V (vector) cells, but this induction was eliminated in H157-ASβ cells, in which RARβ was blocked by RARβ antisense (ASβ) RNA (25) (Figure 5B). The inhibition was at mRNA level since induction of GPRC5A mRNA was eliminated in H157-ASβ (Figure 5C). These suggest that RARβ and RA signaling are essential for GPRC5A expression.

Next, we examined the effect of TNFα on ATRA-induced GPRC5A expression. Immunoblot showed that ATRA treatment induced GPRC5A expression in Calu-1 cells (Figure 5D). Importantly, TNFα treatment significantly inhibited GPRC5A expression at both basal and RA-induced levels (Figure 5D). Consistently, RT-PCR analysis confirmed that TNFα-mediated GPRC5A repression was at mRNA level (Figure 5E). Similar effects were observed in H157 cells, in which GPRC5A at both basal and ATRA-induced levels were significantly repressed by TNFα (Figure 5F). Consistently, co-transfection of p65 suppressed both basal and ATRA-induced luciferase activities in GPRC5A promoter driven-luc reporter in HEK293T cells (Figure 5G). Taken together, RA-induced GPRC5A is repressed by NF-κB signaling.

RelA/p65 interacted with RARα/β and is recruited to the RARE at the GPRC5A promoter. GPRC5A is a target gene of retinoic acid (5, 16, 17); ATRA treatment induces the association of RARα/β to RARE at the GPRC5A promoter (17). To determine the mechanism of NF-κB-mediated GPRC5A repression, we examined the binding of NF-κB at the GPRC5A promoter following TNFα treatment via chromatin immunoprecipitation (ChIP) assay. For comparison, we first examined the recruitment of p65 to IκBα promoter. ChIP analysis showed that p65 was recruited to IκBα promoter after 30 minutes of TNFα treatment, which was followed by enhanced recruitment of RNA polymerase II at 60 and 150 minutes (Figure 6A), indicating that NF-κB induces the assembly of transcription machinery at the IκBα promoter. Next, we examined the effect of NF-κB association at the GPRC5A promoter. Although TNFα treatment induced p65 recruitment to RARE, it significantly suppressed the recruitment of RNA polymerase II to RARE at the GPRC5A promoter at 150-minute interval compared to those at 0 and 30 minutes (Figure 6B). In comparison, the binding of RARα and RXRα to GPRC5A promoter was not changed (Figure 6B). This suggests that TNFα treatment disrupts the assembly of RNA Pol II complex after p65 is recruited to RARE at the GPRC5A promoter.

Since the cis-element RARE is recognized by the trans-element RAR/RXR complex, it raises a question if recruitment of NF-κB to RARE is through the RAR/RXR complex. Next, we examined the interaction between RARα and p65 via immunoprecipitation (IP) assay. IP-immunoblot analysis showed that IP Flag-tagged RARα pull-downed myc-tagged p65 (Figure 6C). This suggests that p65

can interact with RARα. Similarly, IP Flag-tagged RARβ2 and RARβ4 also pull-downed myc-tagged p65 (Figure 6D), indicating that p65 can also interact with RARβ. Thus, NF-κB can interact with the RARα/β complex. Because p65-S276A loss the ability to repress GPRC5A, we then asked if p65-S276A is able to interact with RAR complex. IP-immunoblot analysis showed that, while IP Flag-RARα pull-downed GFP-p65, it failed to pull-down GFP-p65-S276A (Figure 6E). Consistently, immunofluorescent (IF) staining analysis showed that GFP-p65 (green) was highly co-localized with RARα-F (red) in transfected Calu-1 cells; whereas GFP-p65-S276A (green) was mainly located in cytoplasm, not co-localized with RARα-F (red) (Figure 6F). These suggest that the S276 of p65 is essential for interaction with RARα. Taken together, these results suggest that p65, through interaction with RARα/β, is recruited to RARE at the GPRC5A promoter, leading to disrupted the assembly of RNA polymerase II, resulting in suppressed transcription.

Activated NF-κB inhibits H3K9ac in GPRC5A promoter. To determine the general mechanism of GPRC5A repression in NSCLCs, we examined its expression in eight NSCLC cell lines and a normal lung epithelial cell line 16HBE via immunoblot analysis. The results showed that GPRC5A was repressed in most of NSCLC cell lines under normal culture condition (Figure 7A), suggesting that epigenetic repression of GPRC5A is prevalent in NSCLCs (6, 26).

DNA methylation and histone post-translation modification are the major mechanisms involved in epigenetic silence of TSGs (27, 28). To determine the potential role of DNA methylation in GPRC5A repression, we examined the status of DNA methylation in two CpG islands at the GPRC5A promoter in lung cancer tissues and adjacent normal tissues via bisulfite sequencing PCR (BSP). We found that there was no significant difference in GPRC5A promoter methylation between lung cancer and adjacent normal tissues (Supplementary Figure 2). Moreover, we found that overexpression of p65 did not significantly alter the DNA methylation status in Calu-1 cells (Supplementary Figure 3). These suggest that DNA methylation is unlikely to play an important role in GPRC5A repression in NSCLCs.

Next, we asked if post-translation modification of histones plays a role in GPRC5A repression and whether it is induced by NF-κB. ChIP assay showed that TNFα treatment significantly suppressed RNA polymerase II assembly in GPRC5A promoter (Figure 7B); noticeably, acetylated H3K9 (H3K9ac), an active marker of gene expression, was also significantly suppressed in GPRC5A promoter following TNFα treatment. In comparison, H3K27ac at the GPRC5A promoter was not changed following TNFα treatment (Figure 7B). To extend the analysis further, we also examined the effects of NF-κB mutants in histone modification. Over-expression of WT-p65, but not S276A-p65, significantly inhibited the recruitment of RNA polymerase II at the GPRC5A promoter in Calu-1 cells (Figure 7C); importantly, expression of WT p65 inhibited H3K9ac, whereas expression of p65-S276A

did not (Figure 7D). These suggest that NF-κB inhibits both RNA polymerase II assembly and H3K9ac at the GPRC5A promoter. Thus, NF-κB-induced suppression of H3K9ac is involved in epigenetic repression of GPRC5A.

Histone deacetylation but not DNA methylation is mainly responsible for epigenetic repression of GPRC5A in NSCLCs. To determine the full extent of the mechanism underpinning the epigenetic repression of GPRC5A, we examined the roles of DNMT1 inhibitor 5-Aza-2-dc and pan-histone deacetylase (HDAC) inhibitor SAHA in NSCLC cell lines. The results showed that SAHA treatment drastically restored GPRC5A expression in A549, H1975, and Calu-1, whereas 5-Aza-dc had limited effect (Fig. 7E-G). Q-PCR analysis indicated that restoration of GPRC5A expression via various treatment was at the mRNA level (Figure 7H). Treatment with 5-Aza-2-dc had limited effect on GPRC5A mRNA and RARβ mRNA in these NSCLC cell lines (Supplementary Figure 4A-B). These findings further support that NF-κB-induced histone modification, rather than DNA methylation, plays a major role in epigenetic repression of GPRC5A. ChIP assay showed that SAHA treatment significantly enhanced the recruitment of RNA polymerase II to the GPRC5A promoter in Calu-1 cells (Figure 7I), supporting the key role HDACs play in GPRC5A repression. Consistently, SAHA-mediated restoration of GPRC5A was also observed in other NSCLC cell lines, including H460, H1792, HCC827, PC9 and normal cell line 16HBE, although not in H1299 (Figure 7J, supplementary Figure 5). In addition, TNFα treatment suppressed GPRC5A expression in presence of 5-Aza-dc (DNMT inhibitor), but did not in presence of SAHA (Figure 7K). These findings suggest that SAHA abrogates TNFα-mediated repression of GPRC5A; whereas 5-Aza-dc does not. Taken together, NF-κB-mediated histone deacetylation, rather than DNA methylation, is mainly responsible for epigenetic repression of GPRC5A. To determine the biological effects of the histone deacetylation inhibitor in vivo, A549 cells were s.c. inoculated in nude mice, and then treated with SAHA or placebo. SAHA treatment significantly suppressed tumor growth (Supplementary Figure 6A-C) and restored GPRC5A expression compared to the placebo group (Supplementary Figure 6D-E). These results strongly support the notion that histone deacetylation plays a major role in the epigenetic repression of GPRC5A.

To determine the biological roles of GPRC5A-deficiency on lung epithelial cells, we first enhanced the transformed phenotype of mouse tracheal epithelial cells (MTEC) by repeated treatment with tobacco carcinogen NNK (100 pM for ten passages), then performed biological characterization of MTEC-NNK10 cells derived from this treatment. The results showed that MTEC-KO-NNK10 cells formed more and bigger colonies in soft-agar than MTEC-WT-NNK10 cells did (Supplementary Figure 7A-B). This suggests that GPRC5A deficiency enhances the transforming phenotype of lung

epithelial cells. Taken together, NF-κB induces GPRC5A repression via histone deacetylation, whereas GPRC5A repression enhances the oncogenic features in lung epithelial cells.

Discussion

In this study, we showed that NF-κB can function as a transcriptional repressor on GPRC5A expression via a trans-activation independent mechanism. NF-κB-mediated GPRC5A repression is through interaction with RARα/β, resulting in the suppression of RNA polymerase II recruitment in RARE at the GPRC5A promoter. Moreover, NF-κB inhibits histone H3K9ac, rather than induces DNA hyper-methylation, at the GPRC5A promoter. Consistently, SAHA treatment largely restores GPRC5A expression in NSCLC cells, whereas treatment with 5-Aza-dc has little effect. Because Gprc5a-deficience enhances the transformed phenotype of lung epithelial cells, NF-κB-mediated GPRC5A repression contributes to neoplasia of lung epithelial cells.

Chronic inflammation is associated with neoplasia and COPD. Generally, individuals with chronic pulmonary inflammation have an increased incidence of lung cancer development. For examples, COPD patients have about five times more incidence of lung cancer development than normal individuals. Bacterial colonization, particularly non-typeable Haemophilus influenzae (NTHi) (Gram-negative, G- bacteria), is implicated as a cause of airway inflammation in COPD. LPS endotoxin of G- bacteria is a potent inflammatory inducer. Extrinsic lung inflammation induced by repeated administration of NTHi, in combination with tobacco carcinogen NNK, enhanced lung tumorigenesis in *Gprc5a*-ko mice (29). These observations strongly suggest that chronic inflammation promotes lung tumorigenesis.

NF-κB is a well-known transcriptional activator (30). However, little is known about its role as a transcriptional repressor. Previously, it was reported that RelA/p65 can act as a transcriptional repressor to repress metastasis suppressor gene BRMS1 (breast cancer metastasis suppressor 1). Mechanistically, p65 binds the specific κB binding site at the BRMS1 promoter and recruit DNMT-1 (DNA (cytosine-5)-methyltransferase 1), which mediates DNA methylation in CpG islands located at the promoter region, resulting in epigenetic repression (21). Nevertheless, the mechanism of NF-κB-mediated BRMS1 repression is via induction of DNA methylation, which is distinct from the repression of GPRC5A via histone deacetylation found in this study.

Retinoic acid (RA) signaling plays an important role in the regulation of cell proliferation, differentiation and homeostasis. RA exerts its role by interacting with nuclear retinoic acid receptors (RAR) and retinoic X receptors (RXRs). GPRC5A is a RA target. Thus, the status of RARs are crucial for GPRC5A expression. Of note, RARβ expression is often lost or reduced in a large percentage of lung cancer (31, 32). For example, methylation of the RARβ2 promoter is found in 40% of NSCLCs. Thus, it raises an interesting question whether hyper-methylation of RARβ plays an important role in GPRC5A repression. Although 5-Aza-2-dc treatment has minor increase of RARβ mRNA in NSCLC cells, it does not have any effect on the level of GPRC5A mRNA, consistent with the observation of

no DNA methylation found at the GPRC5A promoter. In contrast, SAHA treatment greatly elevates the levels of GPRC5A mRNA, supporting the critical role of H3K9ac in regulating GPRC5A expression. Although combination of 5-Aza-dc and SAHA further increased GPRC5A mRNA, the additive effect of 5-Aza-dc on GPRC5A expression is likely mediated by the minor increase of RARβ, which indirectly increases GPRC5A expression.

NF-κB has been found to crosstalk with other signaling pathways, including EGFR (33), p53 (34), HIF1α (35), glucocorticoid signaling (36). It was shown that vitamin A deficiency enhances inflammatory response or NF-κB activation *in vivo*, whereas administration of ATRA reduces inflammatory response or NF-κB activation in animal models (37). These suggest that vitamin A or RA signaling inhibits NF-κB signaling. Moreover, the p50 and p65 components of NF-κB were shown to bind to retinoid X receptor (RXR) in a ligand-independent manner, and RXR can inhibit NF-κB activation in a ligand-dependent manner (38). These suggest that there is a crosstalk between NF-κB and RA signaling. Retinoids play a fundamental role in development and homeostasis (39), whereas disruption of the RA signaling pathway is implicated in neoplasia or cancers (40) (41). Conversely, activation of RA signaling is used as a strategy for cancer prevention and therapy (42). GPRC5A was originally cloned as retinoic acid-inducible gene 1 (RAIG1) (5). A RA response element (RARE) at the GPRC5A promoter is critical for its expression (17). Thus, GPRC5A functions as a key mediator of RA signaling for differentiation or homeostasis in lung tissue. Presumably, the mutually inhibitory effects between NF-κB and RARα/β signaling represent a molecular switch at the crossroad of cell fate determination between differentiation and dedifferentiation.

Previously, we showed that *Gprc5a*-ko leads to increased NF-κB activation in lung epithelium, which is associated with lung tumorigenesis in mouse model. *Gprc5a*-ko mice are susceptible to pulmonary inflammation and LPS-induced acute lung injury (10-11). However, the regulatory role of Gprc5a-deletion on NF-κB activation in lung epithelial cells appears to be indirect since the effects are stronger in vivo than in vitro. In addition, the effect of GPRC5A-knockdown on the NF-κB activation in human lung epithelial cells is not as strong as that in mouse lung epithelial cells. It is likely that multiple mechanisms are involved in the cross-talk between GPRC5A and NF-κB.

Taken together, we find that NF-κB functions as a transcriptional repressor to suppress GPRC5A expression. NF-κB interacts with RARα/β via its transactivation domain, which disrupts the assembly of RAR-mediated RNA polymerase II complex at the GPRC5A promoter, leading to its transcriptional repression. Concurrently, NF-κB induces epigenetic repression via H3K9ac histone deacetylation at the GPRC5A promoter (Figure 8). Importantly, this epigenetic mechanism of GPRC5A repression is prevalent in NSCLCs. We propose that NF-κB-mediated GPRC5A repression contributes to dedifferentiation or neoplasia in lung epithelial cells.

Methods

Cells, reagents and clinical samples. Multiple types of NSCLC cell lines were used in this work including bronchioloalveolar carcinoma (H322), mucoepidermoid pulmonary carcinoma (H292G), epidermoid carcinoma (Calu-1), large cell lung cancer (H460), adenocarcinoma (H157, PC9, HCC827, H1792, H1975) and lung carcinoma (A549, H1299). All NSCLC tumor cell lines were obtained from ATCC. The A549 and Calu-1 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. All other tumor cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. 16HBE, a normal human bronchial epithelial cell line, was maintained in DMEM medium supplemented with 10% fetal bovine serum. Wild and Gprc5a-ko MTEC (mouse tracheal epithelial cells) cells were obtained from normal tracheal tissue of 3-week-old WT and Gprc5aknockout (ko) mice (C57 BL/6 129sv) as described previously (10, 12). Cells were cultured with keratinocyte-SFM supplemented with EGF (5 ng/mL) and bovine pituitary extract (50 mg/mL; Invitrogen). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St Louis, MO); TNFα was purchased from R&D (Minneapolis, MN); Doxycycline were purchased from Selleckchem (Houston, TX); Antibodies against H3K9ac (ab4441), myc-tag for ChIP (ab9232) were purchased from Abcam (Cambridge, MA); Antibodies against Gprc5a (sc-98885), IκBα (sc-371), GAPDH (sc-365062), RARα (C-20), RARβ (C-19) and GFP (sc-8334) were purchased from Santa Cruz (Santa Cruz, CA). NF-κB p65 NLS antibody (for IHC, NBP2-24541) was purchased from Novus (Littleton, CO), Antibodies against p-p65 (S536; #3033), H3K27Ac (#8173), β-actin conjugated with HRP (#12262) were purchased from Cell Signaling Technology (Beverley, MA). p-p65 (S276) antibody (D155005) was from BBI LIFE SCIENCES CORPORATION (Shanghai, China); RNA polymerase II antibody (#05-623B) was from Millipore; Antibodies against myc-tag (562-5), Flag-tag (PM020) were purchased from MBL international corporation (Woburn, MA); GPRC5A antibody was generated by Abmart company (Shanghai, China). All human clinical samples were provided by Shanghai Chest Hospital (Shanghai, China) and all informed consents were obtained.

Animal experiment. NF-κB-driven luciferase transgenic mice were administrated with LPS through atomization inhalation (500 μg in 5 ml for each cage per day). D-luciferin was injected intraperitoneally 30 minutes after LPS treatment; another two hours later, fluorescence imaging was performed to verify the activation of NF-κB. Mice were sacrificed at day 4, 6, 8, and lungs were analyzed via western blot and Q-PCR assays to examine the protein and mRNA levels of Gprc5a.

Luciferase Reporter Assay. Cells were grown to 50% confluence in 24-well plate and then co-transfected with reporter gene constructs (GPRC5A-luc or NF-κB-luc) and pcDNA3.1-p65 and its mutants. Plasmid pRL-TK (Promega) was used as internal control in all transfection assays. All

transfections were done with Lipofectamine 2000 (Invitrogen) according to the instruction of manufacture (43). Cell extracts were prepared 48 hours after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). All experiments were performed three times in triplicate.

Immunoprecipitation assay and Western blot analysis. Cells or tissue were lysed with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris (pH 8.0), 25 mM NaF, 2 mM Na_3VO_4, 5 mM PMSF and 2 mg/ml of aprotinin) (44). Immunoprecipitation and Western blot were performed as described previously (45, 46). Briefly, 2 μg Flag antibody were used for 500μg total whole cell lysate for each immunoprecipitation. For western blot, whole cell lysate containing 30 μg whole protein was mixed with 2x SDS-PAGE reduced loading buffer and boiled at 95℃ for 5 min for per gel well. Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membrane for western blot. Non-specific binding to antibodies were blocked with 5% non-fat milk at room temperature for 1h. Primary antibodies were incubated at 4℃ overnight. To remove the residual primary antibody, membranes were washed three times in TBST (0.05% Tween-20) while agitating, 5 minutes for per wash. Second antibody conjugated with HRP were incubated at room temperature for 1 hour and washed three times in TBST while agitating. ECL kit (Millipore) was used to detection.

Duplex Reverse transcription PCR and Quantitative real time PCR. Total RNA was isolated using the RNAsimple Total RNA Kit (TIANGEN) according to the manufacturer's instructions. Reverse transcription was performed using FastQuant RT Kit (TIANGEN) according to the manufacturer's protocol. For Duplex RT-PCR, 0.5 μl cDNA from each sample was amplified with the GPRC5A primers (6) plus β-actin competition primers from Ambion (Austin, TX, USA) in high fidelity PCR master mix (Roche applied science, Indianapolis, IN) according to the manufacturer's instructions. For specific quantitative real-time PCR, experiments were performed using SuperReal PreMix Plus SYBR Green Kit (TIANGEN) according to the manufacturer's protocol. All primers used for quantitative real time PCR were listed in Supplementary Table 1-2.

Immunohistochemistry staining. Tissue samples from human lung cancer, chronic obstructive pulmonary disease (COPD) and adjacent normal tissues were stained with anti-GPRC5A (1:200 dilution) and anti-p65 NLS (1:100 dilution) antibodies, and each sample was scored by an H-score method that combines the values of immune-reaction intensity and the percentage of cell staining as described previously (47). Pearson correlation analysis was used to analyze the relationship between GPRC5A expression and activated p65; statistical significance was defined as $p < 0.05$.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay were performed by using ChIP kits (#9003, Cell signaling) according to the manufacturer's instructions. The primers used for ChIP were listed in Supplementary Table 3.

Immunofluorescence. GFP-p65 or GFP-p65-S276A mutant were co-transfected with RARα-Flag to Calu-1 cells, RARα-Flag was stained with Flag antibody (sigma, M2), The procedure of immunofluorescence was done as described previously (48).

Statistical analysis. Differences between experimental groups were analyzed by paired Student's t-test. All results are presented as mean SD and represented histogram or images were selected based on the average values, $p<0.05$ was considered significant (*$p<0.05$; **$p< 0.01$; ***$p< 0.001$).

Author contributions

HS, XY, YL, and SZ designed, performed, and analyzed experiments, and co-wrote the article. DX, BJ, TW, BS, JX, WG, KL, MH, YK, JL, TZ, YW, FY helped with experiments and provided critical tools and advice. BPZ, QW, JD supervised and co-wrote the manuscript.

Acknowledgments

This work was supported by NSFC (China) (No. 81620108022, 91129303, 91729302 and 81572759 to J. Deng; No. 91129733, 81502702 to Q. Wang; No. 81902313 to HY. Song, No. 82002941 to B. Sun, No. 31900441 to J. Du, No. 82072570 to F. Yao); by the Fundamental Research Funds for the Central Universities (to HY Song) and by the Youth Foundation of Shanghai Municipal Commission of Health and Family Planning (No. 20164Y0261 to HY. Song).

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Coussens LM, and Werb Z. Inflammation and cancer. *Nature.* 2002;420(6917):860-7.
2. Cho WCS, et al. The role of inflammation in the pathogenesis of lung cancer. *Expert Opinion on Therapeutic Targets.* 2011;15(9):1127-37.
3. Houghton AM. Mechanistic links between COPD and lung cancer. *Nature Reviews Cancer.* 2013;13(4):233-45.
4. Gordon S. Alternative activation of macrophages. *Nature Reviews Immunology.* 2003;3(1):23-35.
5. Tao Q, et al. Characterization of the murine orphan G-protein-coupled receptor gene Rai3 and its regulation by retinoic acid. *Genomics.* 2004;83(2):270-80.
6. Tao Q, et al. Identification of the retinoic acid-inducible Gprc5a as a new lung tumor suppressor gene. *J Natl Cancer Inst.* 2007;99(22):1668-82.
7. Sporn MB. A new tumor suppressor gene, selective for lung cancer. *J Natl Cancer Inst.* 2007;99(22):1654-5.
8. Zhong S, et al. Lung Tumor Suppressor GPRC5A Binds EGFR and Restrains Its Effector Signaling. *Cancer Res.* 2015;75(9):1801-14.
9. Wang J, et al. GPRC5A suppresses protein synthesis at the endoplasmic reticulum to prevent radiation-induced lung tumorigenesis. *Nat Commun.* 2016;7:11795.
10. Deng J, et al. 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;3(4):424-37.
11. Liao Y, et al. Gprc5a-deficiency confers susceptibility to endotoxin-induced acute lung injury via NF-kappaB pathway. *Cell Cycle.* 2015;14(9):1403-12.
12. Chen Y, et al. Gprc5a deletion enhances the transformed phenotype in normal and malignant lung epithelial cells by eliciting persistent Stat3 signaling induced by autocrine leukemia inhibitory factor. *Cancer Res.* 2010;70(21):8917-26.
13. Song H, et al. GPRC5A deficiency leads to dysregulated MDM2 via activated EGFR signaling for lung tumor development. *Int J Cancer.* 2019;144(4):777-87.
14. Kadara H, et al. A Gprc5a tumor suppressor loss of expression signature is conserved, prevalent, and associated with survival in human lung adenocarcinomas. *Neoplasia.* 2010;12(6):499-505.
15. Liu S-l, et al. Repression of G protein-coupled receptor family C group 5 member A is associated with pathologic differentiation grade of oral squamous cell carcinoma. *Journal of Oral Pathology & Medicine.* 2013;42(10):761-8.
16. Cheng Y, and Lotan R. Molecular cloning and characterization of a novel retinoic acid-inducible gene that encodes a putative G protein-coupled receptor. *J Biol Chem.* 1998;273(52):35008-15.
17. Ye X, et al. Mechanisms underlying the induction of the putative human tumor suppressor GPRC5A by retinoic acid. *Cancer Biol Ther.* 2009;8(10):951-62.
18. Chen LF, et al. Duration of nuclear NF-kappa B action regulated by reversible acetylation. *Science.* 2001;293(5535):1653-7.
19. Yang F, et al. IKK beta plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. *Journal of Immunology.* 2003;170(11):5630-5.
20. Schmitz ML, et al. NF-kappa B: A multifaceted transcription factor regulated at several levels. *Chembiochem.* 2004;5(10):1348-58.
21. Liu Y, et al. Phosphorylation of RelA/p65 promotes DNMT-1 recruitment to chromatin and represses transcription of the tumor metastasis suppressor gene BRMS1. *Oncogene.* 2012;31(9):1143-54.
22. Hoffmann A, et al. Transcriptional regulation via the NF-kappa B signaling module. *Oncogene.* 2006;25(51):6706-16.
23. Gilmore TD. Introduction to NF-kappa B: players, pathways, perspectives. *Oncogene.* 2006;25(51):6680-4.
24. Ye X, et al. Mechanisms underlying the induction of the putative human tumor suppressor GPRC5A by retinoic acid. *Cancer Biology & Therapy.* 2009;8(10):951-62.
25. Sun SY, et al. Evidence that retinoic acid receptor beta induction by retinoids is important for tumor cell growth inhibition. *The Journal of biological chemistry.* 2000;275(22):17149-53.
26. Fujimoto J, et al. G-Protein Coupled Receptor Family C, Group 5, Member A (GPRC5A) Expression Is Decreased in the Adjacent Field and Normal Bronchial Epithelia of Patients with Chronic

Obstructive Pulmonary Disease and Non-Small-Cell Lung Cancer. *Journal of Thoracic Oncology.* 2012;7(12):1747-54.
27. Liu Y, et al. Phosphorylation of RelA/p65 promotes DNMT-1 recruitment to chromatin and represses transcription of the tumor metastasis suppressor gene BRMS1. *Oncogene.* 2012;31(9):1143-54.
28. Wang Z, et al. Epigenetic silencing of the 3p22 tumor suppressor DLEC1 by promoter CpG methylation in non-Hodgkin and Hodgkin lymphomas. *J Transl Med.* 2012;10:209.
29. Barta P, et al. Enhancement of lung tumorigenesis in a Gprc5a Knockout mouse by chronic extrinsic airway inflammation. *Molecular Cancer.* 2012;11.
30. Zhang D, et al. Combined prognostic value of both RelA and IkappaB-alpha expression in human non-small cell lung cancer. *Ann Surg Oncol.* 2007;14(12):3581-92.
31. Houle B, et al. Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. *Proceedings of the National Academy of Sciences of the United States of America.* 1993;90(3):985-9.
32. Picard E, et al. Expression of retinoid receptor genes and proteins in non-small-cell lung cancer. *Journal of the National Cancer Institute.* 1999;91(12):1059-66.
33. Shostak K, and Chariot A. EGFR and NF-kappaB: partners in cancer. *Trends Mol Med.* 2015;21(6):385-93.
34. Goyal U, and Ta M. p53-NF-kappaB Crosstalk in Febrile Temperature-Treated Human Umbilical Cord-Derived Mesenchymal Stem Cells. *Stem Cells Dev.* 2019;28(1):56-68.
35. Qiu S, et al. Lipocalin-2 protects against renal ischemia/reperfusion injury in mice through autophagy activation mediated by HIF1alpha and NF-kappab crosstalk. *Biomed Pharmacother.* 2018;108:244-53.
36. Ling J, and Kumar R. Crosstalk between NFkB and glucocorticoid signaling: a potential target of breast cancer therapy. *Cancer Lett.* 2012;322(2):119-26.
37. Austenaa LMI, et al. Vitamin A status significantly alters nuclear factor-kappa B activity assessed by in vivo imaging. *Faseb Journal.* 2004;18(9):1255-+.
38. Na SY, et al. Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NF kappa B. *Journal of Biological Chemistry.* 1999;274(12):7674-80.
39. Ross SA, et al. Retinoids in embryonal development. *Physiological Reviews.* 2000;80(3):1021-54.
40. Wang S, and Moise AR. Recent insights on the role and regulation of retinoic acid signaling during epicardial development. *Genesis.* 2019;57(7-8).
41. de The H, et al. The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell.* 1991;66(4):675-84.
42. Abaza Y, et al. Long-term outcome of acute promyelocytic leukemia treated with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab. *Blood.* 2017;129(10):1275-83.
43. Deng J, et al. beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. *Cancer cell.* 2002;2(4):323-34.
44. Lin Y, et al. The SNAG domain of Snail1 functions as a molecular hook for recruiting lysine-specific demethylase 1. *EMBO J.* 2010;29(11):1803-16.
45. Zhou BP, et al. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nature cell biology.* 2004;6(10):931-40.
46. Wu Y, et al. Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion. *Cancer cell.* 2009;15(5):416-28.
47. Deng JO, et al. beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. *Cancer Cell.* 2002;2(4):323-34.
48. Zhou BHP, et al. Dual regulation of Snail by GSK-3 beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nature Cell Biology.* 2004;6(10):931-+.

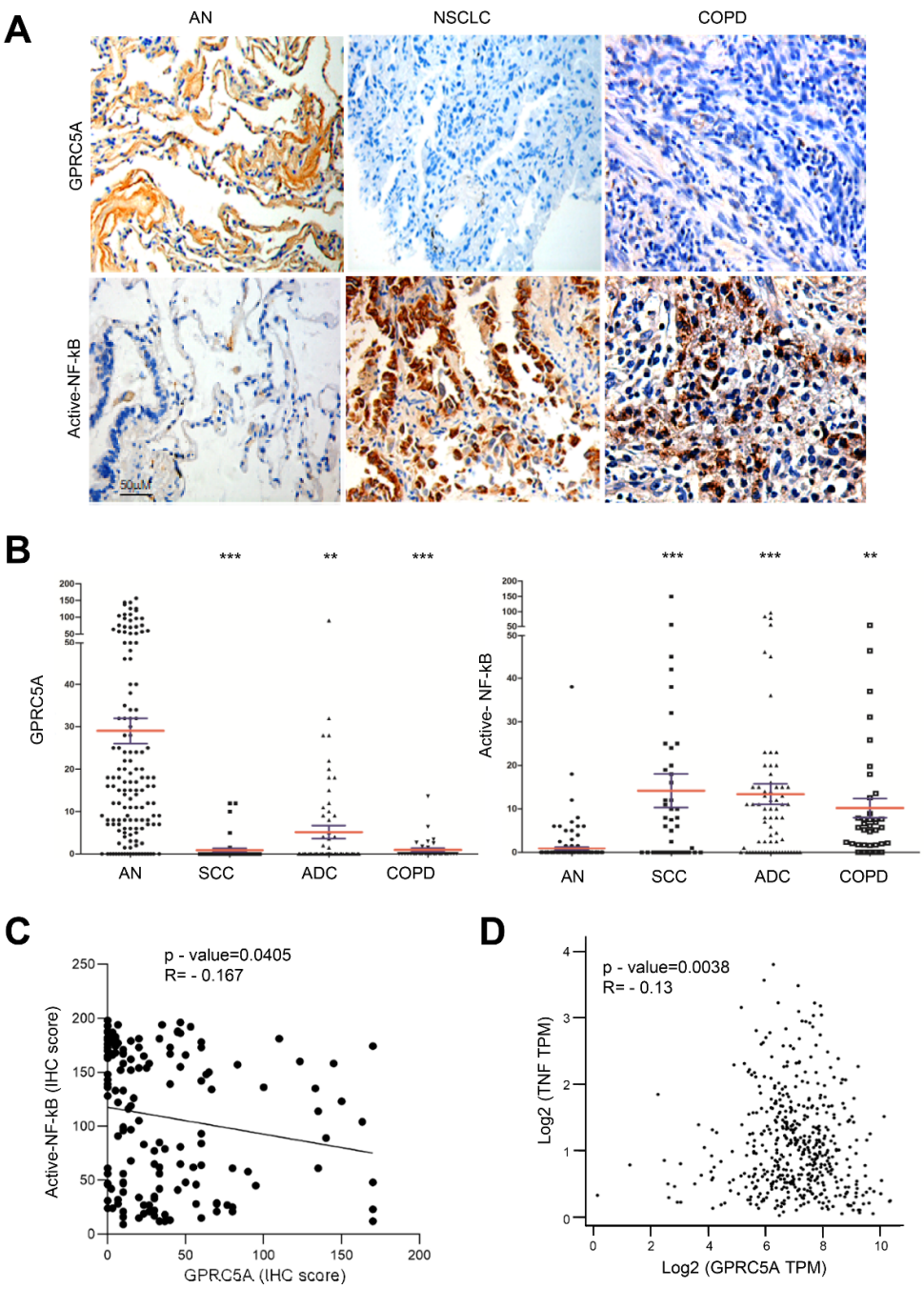


Figure 1. Active NF-κB is associated with GPRC5A repression in human NSCLC and COPD clinical samples. (**A**) Representative images of IHC staining for GPRC5A and activated NF-κB in human clinical samples. (**B**) Quantification of the IHC staining is represented as IHC scores of GPRC5A and activated NF-κB in human clinical samples; data are presented as the mean ± SD. (**C**) IHC scores of GPRC5A and activated NF-κB in human NSCLC clinical samples and adjacent normal tissues were analyzed by Pearson correlation. (**D**) Pearson correlation analysis of mRNA expression of TNFα and GPRC5A in human lung adenocarcinoma (LUAD) clinical samples from TCGA database. AN, adjacent normal; NSCLC, non-small cell lung cancer; COPD, chronic obstructive pulmonary disease; SCC, squamous cell carcinoma; ADC, adenocarcinoma. *p< 0.05; **p< 0.01; ***p< 0.001.

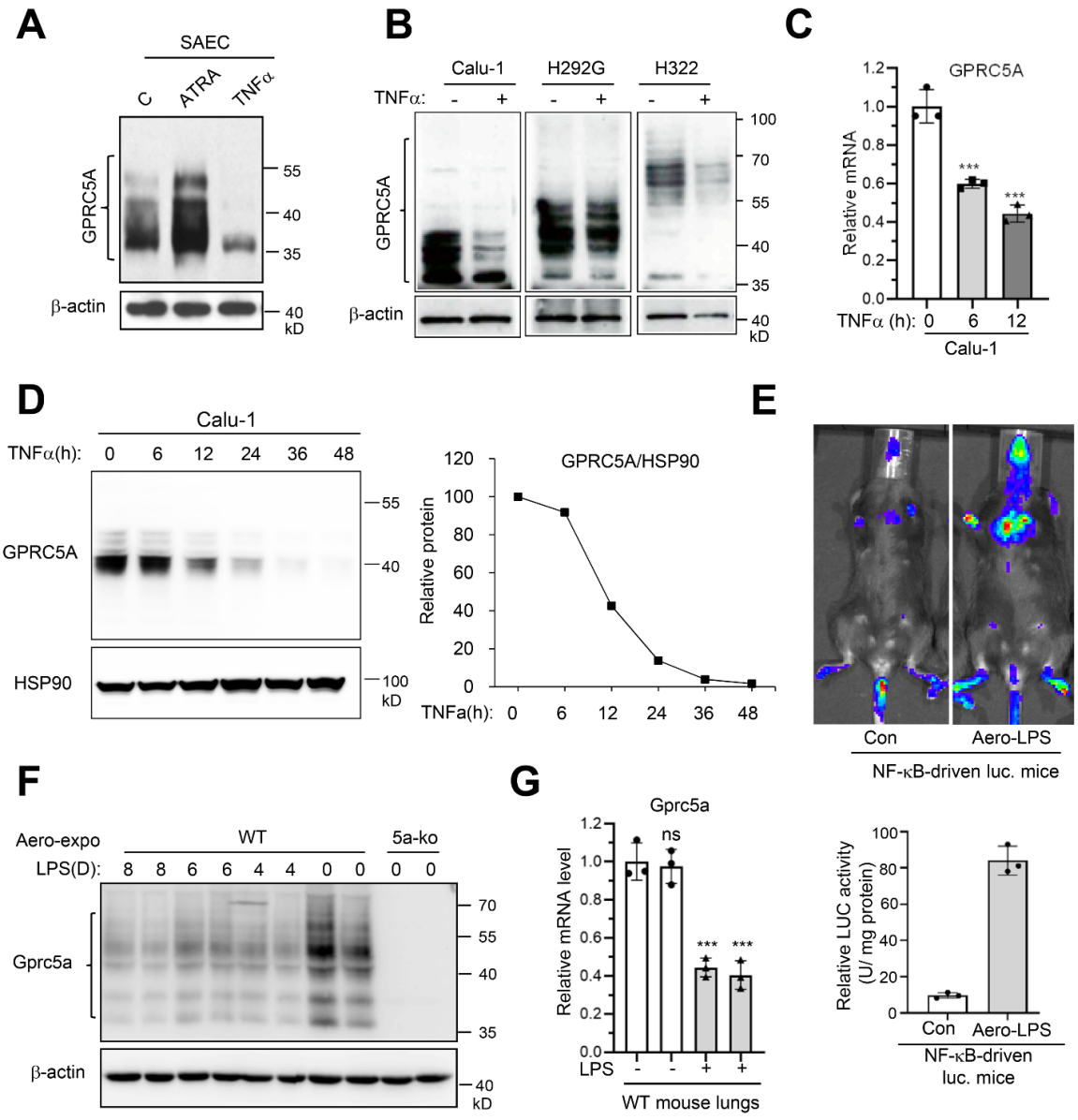


Figure 2. Inflammatory signaling inhibits GPRC5A both in vitro and in vivo. (**A**) Human small airway epithelial cells (SAEC) was treated with ATRA (1 μM), TNFα (10 ng/ml) or CSC (1 μM) for 24h, and the protein level of GPRC5A was analyzed by western blotting. (**B**) Calu-1, H292G, H322 was treated with TNFα (10 ng/ml) for 24 hours and the protein level of GPRC5A was determined by western blotting. (**C**, **D**) Calu-1 cells were treated with TNFα (10 ng/ml) for indicated times. The levels of mRNA (C) and protein (D) of GPRC5A was determined by quantitative PCR and western blotting, respectively. Data are presented as the mean ± SD. (**E**) Represent data of luciferase activity of NF-κB driven luciferase transgenic mice treated with placebo or LPS (500 μg/5 ml) for 30 min through inhalation. (**F**) Mice treated with LPS through inhalation for various days and the mouse lung tissue was analyzed for Gprc5a expression via western blotting, data are presented as the mean ± SD. (**G**) Mouse was treated with LPS through inhalation for 4 days, and lung tissue was collected, the mRNA level of Gprc5a was determined by quantitative PCR. All data are presented as mean ± SD from three independent experiments with duplicates and analyzed by 2-tailed Student's t-test; ns, not significant; *p<0.05; **p< 0.01; ***p<0.001.

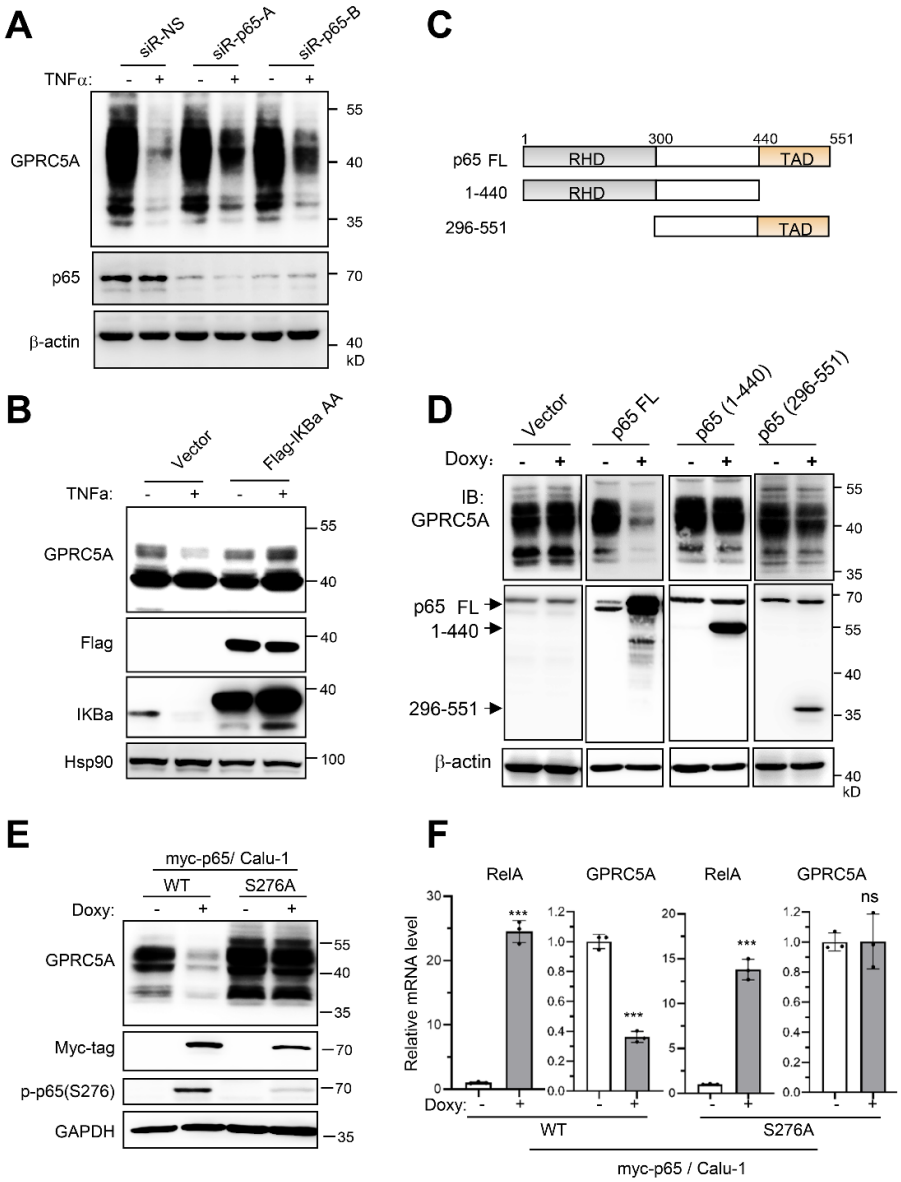


Figure 3. Transcription activation domain of NF-κB is required for GPRC5A repression. (**A**) Small interfering RNA (siRNA) targeting p65 and scramble control siRNA were transfected to Calu-1 cells, then treated with or without TNFα. The protein levels of GPRC5A and p65 were determined by western blotting. (**B**) Calu-1 cells were transfected with plasmid overexpressing IκBα AA mutant (S32A, S36A) or vector control, then treated with or without TNFα, GPRC5A and IκBα protein levels were determined with specific antibodies through western blotting. (**C**) Schematic representation of wild type and truncation mutant of RelA/p65. (**D**) Calu-1 transfectants harboring vector control or inducible expression of full-length and truncated p65 were established; cells were treated with doxycycline (300 ng/ml) for 24 hours, and GPRC5A protein levels were analyzed by western blotting. These blots were run in separated gels performed parallelly with equal loading (please see Uncropped/Unedited gel document). (**E**, **F**) Calu-1 cells with inducible expression wild-type p65 and S276A mutant were treated with doxycycline (300 ng/ml) for 24 hours, GPRC5A protein and mRNA levels were determined by western blotting (E) and quantitative PCR (F) respectively. Data are presented as mean ± SD from three independent experiments with duplicates and analyzed by 2-tailed Student's t-test, *p<0.05; **p<0.01; ***p< 0.001.

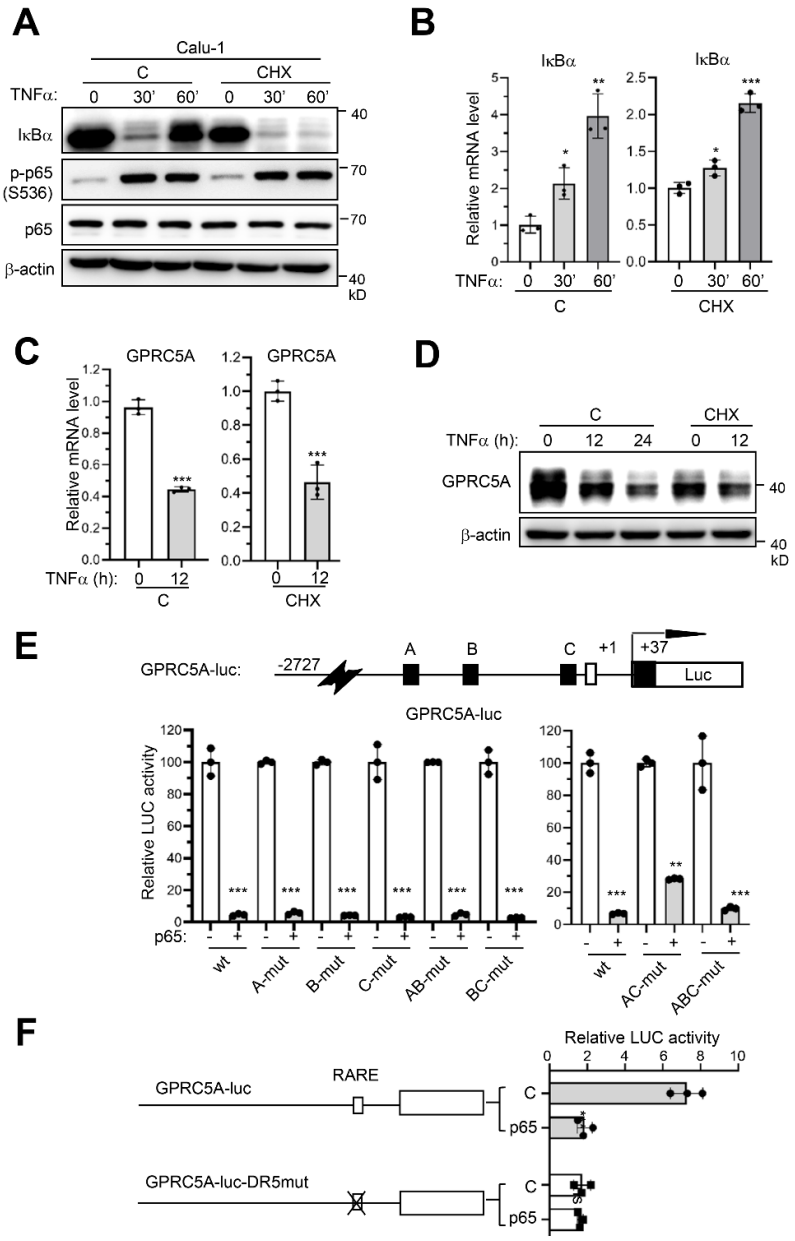


Figure 4. NF-κB-mediated repression of GPRC5A is independent of transcription activation. (**A**, **B**) Calu-1 cells were treated with TNFα and CHX separately or in combination, IκBα protein and mRNA levels were determined by western blotting and quantified with image J (A) and quantitative PCR (B) respectively. Data are presented as the mean ± SD. (**C**, **D**) Calu-1 cells were treated with TNFα and CHX separately or in combination, GPRC5A mRNA and protein levels were determined by quantitative PCR (C) and western blotting and quantified with image J (D) respectively. Data are presented as the mean ± SD. (**E**) Three NF-κB binding sites (designated as A, B and C) on GPRC5A promoter-luc plasmid were mutated individually or in combination. The repression effect of p65 was determined by luciferase assay. (**F**) RAR response element (RARE) at the GPRC5A promoter-luc plasmid was mutated and the repression effect of p65 was determined by luciferase assay. Data are presented as mean ± SD from three independent experiments with duplicates and analyzed by 2-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001.

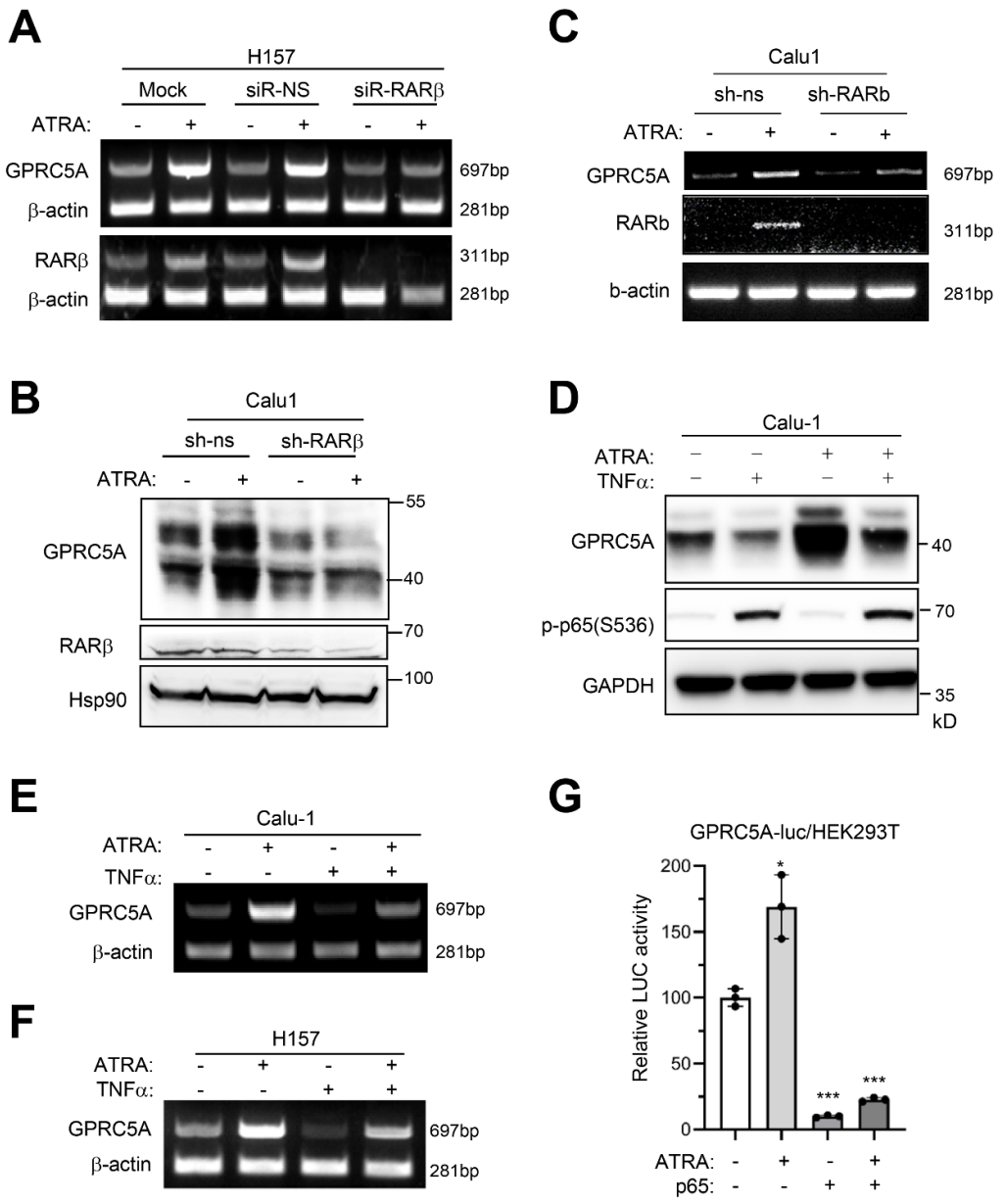


Figure 5. NF-κB inhibits RA induced GPRC5A expression. (**A**) RARβ in H157 cells was knocked down by small interfering RNA followed by treatment with or without all-trans retinoic acid (ATRA). The mRNA levels of RARβ and GPRC5A were determined by RT-PCR and quantified by image J. (**B**, **C**) RARβ in H157 cells was knocked down by RARβ antisense (ASβ) RNA followed by treatment with or without all-trans retinoic acid (ATRA). Protein and mRNA levels of RARβ and GPRC5A were determined by western blotting (B) and RT-PCR and quantified by image J (C), respectively. (**D**, **E**) Calu-1 cells was treated with TNFα and ATRA separately or in combination, and the GPRC5A protein and mRNA levels were determined by western blotting (D) and RT-PCR and quantified by image J (E). (**F**) H157 cells was treated with TNFα and ATRA separately or in combination. GPRC5A mRNA levels were determined by RT-PCR and quantified by image J. (**G**) 293T cells was transfected with GPRC5A-luc and p65 plasmids and treated with or without ATRA. The p65 repression effect was determined by luciferase assay. Data are presented as mean ± SD from three independent experiments with duplicates and analyzed by 2-tailed Student's t-test, *p<0.05; **p<0.01; ***p<0.001.

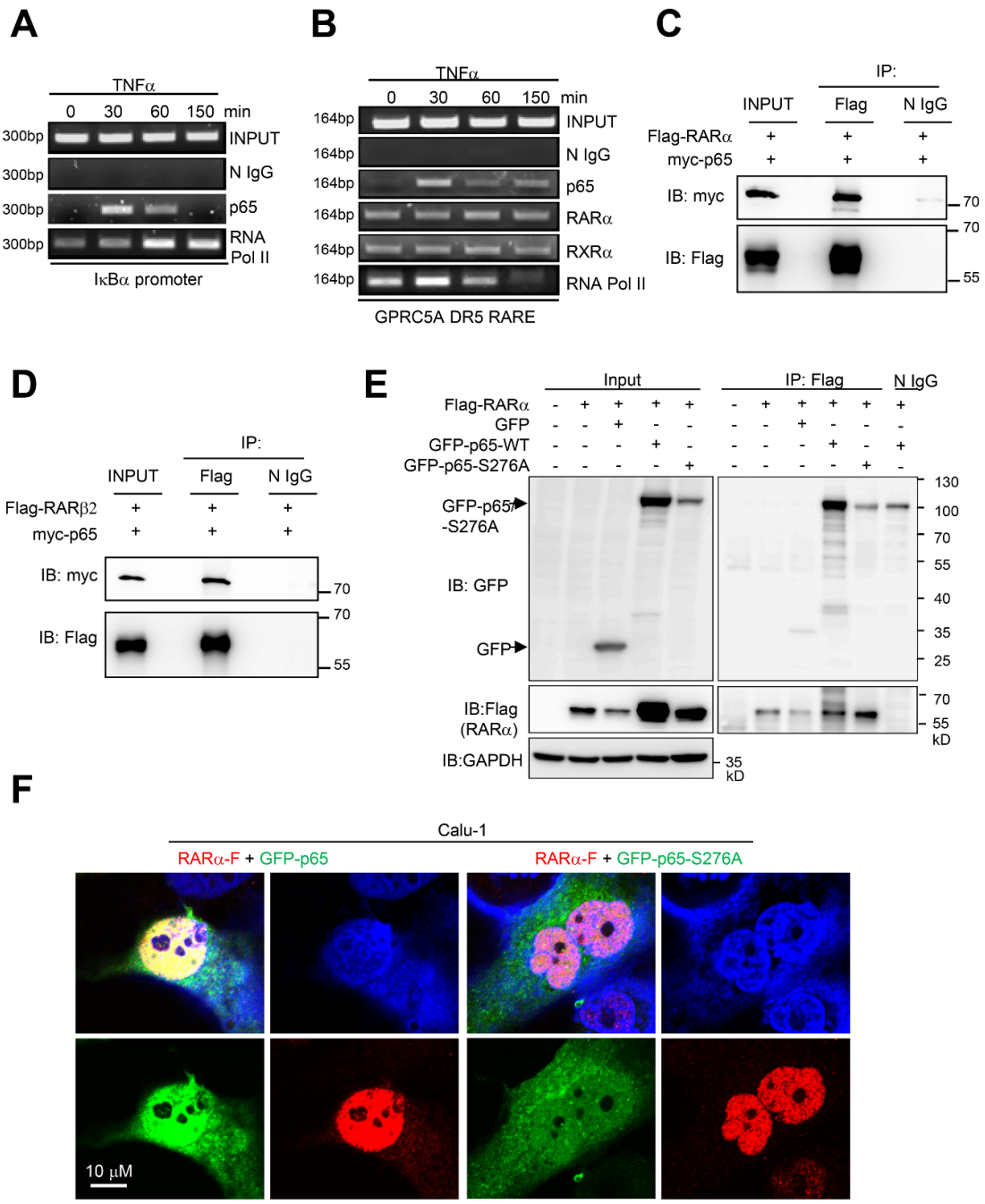


Figure 6. NF-κB subunit p65 is recruited to the promoter of GPRC5A via physically interacted with RAR. (**A**, **B**) Calu-1 cells were treated with TNFα (10 ng/ml) for various time points, the change of p65 and other proteins as indicated in Figure binding to IκBα promoter (A) and GPRC5A promoter (B) were analyzed by chromatin immunoprecipitation assay using corresponding specific antibodies; input as positive control and normal IgG (N IgG) as negative control. (**C**, **D**) The interaction of p65 with RARα (C) and RARβ2/β4 (D) were determined by immunoprecipitation assay. (**E**) RARα-Flag expressing plasmid were co-transfected with GFP/GFP-p65/GFP-p65-S276A mutant to 293T cells, after 48 hours, cells were lysed by RIPA and immunoprecipitation assay were performed to detect the interaction between RARα and wild type RelA/p65 or S276A mutant. (**F**) Representative images of IF analysis of Calu-1 cells transfected with RARα-Flag plus GFP-p65 or GFP-p65-S276A as indicated.

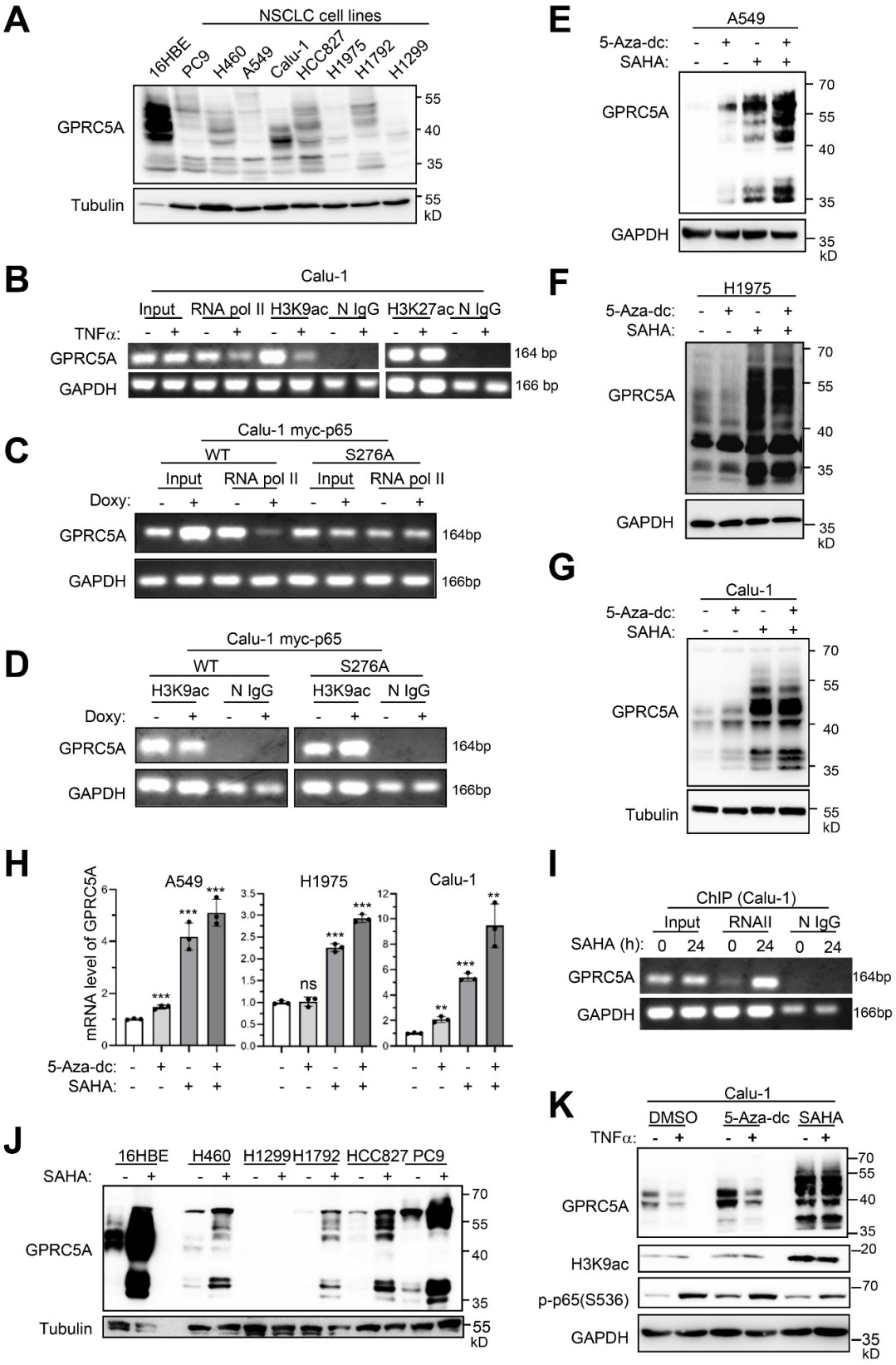

A
NSCLC cell lines
16HBE
PC9
H460
A549
Calu-1
HCC827
H1975
H1792
H1299
GPRC5A
Tubulin
55
40
35
55
kD
B
Calu-1
Input
RNA pol II
H3K9ac
N IgG
H3K27ac
N IgG
TNFα:
GPRC5A
GAPDH
164 bp
166 bp
C
Calu-1 myc-p65
WT
S276A
Input
RNA pol II
Input
RNA pol II
Doxy:
GPRC5A
GAPDH
164bp
166bp
D
Calu-1 myc-p65
WT
S276A
H3K9ac
N IgG
H3K9ac
N IgG
Doxy:
GPRC5A
GAPDH
164bp
166bp
E
A549
5-Aza-dc:
SAHA:
GPRC5A
GAPDH
70
55
40
35
35
kD
F
H1975
5-Aza-dc:
SAHA:
GPRC5A
GAPDH
70
55
40
35
35
kD
G
Calu-1
5-Aza-dc:
SAHA:
GPRC5A
Tubulin
70
55
40
35
55
kD
H
mRNA level of GPRC5A
A549
H1975
Calu-1
***
***
***
ns
***
***
**
***
**
5-Aza-dc:
SAHA:
I
ChIP (Calu-1)
Input
RNAII
N IgG
SAHA (h):
0
24
0
24
0
24
GPRC5A
GAPDH
164bp
166bp
J
16HBE
H460
H1299
H1792
HCC827
PC9
SAHA:
GPRC5A
Tubulin
70
55
40
35
55
kD
K
Calu-1
DMSO
5-Aza-dc
SAHA
TNFα:
GPRC5A
H3K9ac
p-p65(S536)
GAPDH
70
55
40
35
20
70
35
kD

Figure 7. NF-κB-mediated GPRC5A suppression is associated with epigenetic alteration. (**A**) GPRC5A protein expression level in multiple human NSCLC cell lines and normal human bronchial epithelial cell line (16HBE) were analyzed by western blotting. (**B**) Calu-1 cells treated with or without TNFα (10 ng/ml) for 12 hours, binding of RNA polymerase II and the histone modification at the GPRC5A promoter were analyzed by ChIP using specific antibody. (**C**, **D**) Calu-1 cells with inducible expressing of WT and S276A mutant p65 treated with doxycycline (300 ng/ml) for 12 hours, the change of RNA polymerase II binding (C) and histone modification (H3K9ac) at the GPRC5A promoter (D) were analyzed by ChIP. Input as positive control and normal IgG (N IgG) as negative control. (**E-H**) A549, H1975 and Calu-1 cells were treated with 5-Aza-dc (1 μM, 4 days) or SAHA (2.5 μM, 24 hours) individually or in combination, GPRC5A protein (E-G) and mRNA levels (H) were analyzed via western blotting and quantitative PCR. Data are presented as the mean ± SD. (I) Calu-1 cells were treated with or without SAHA (2.5 μM) for 12 hours, RNA polymerase II binding at the GPRC5A promoter was analyzed by ChIP. (**J**) 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 protein levels were analyzed by western blotting. (**K**) Calu-1 cells were pretreated with DMSO (as vehicle control), 5-Aza-dc (1 μM, 3 days) or SAHA (2.5 μM, 3 hours) followed by TNFα (10ng/ml) treatment for additional 24 hours. GPRC5A protein levels were analyzed by western blotting. All data are presented as mean ± SD from three independent experiments with duplicates and analyzed by 2-tailed Student's t-test, *$p<0.05$; **$p<0.01$; ***$p<0.001$.

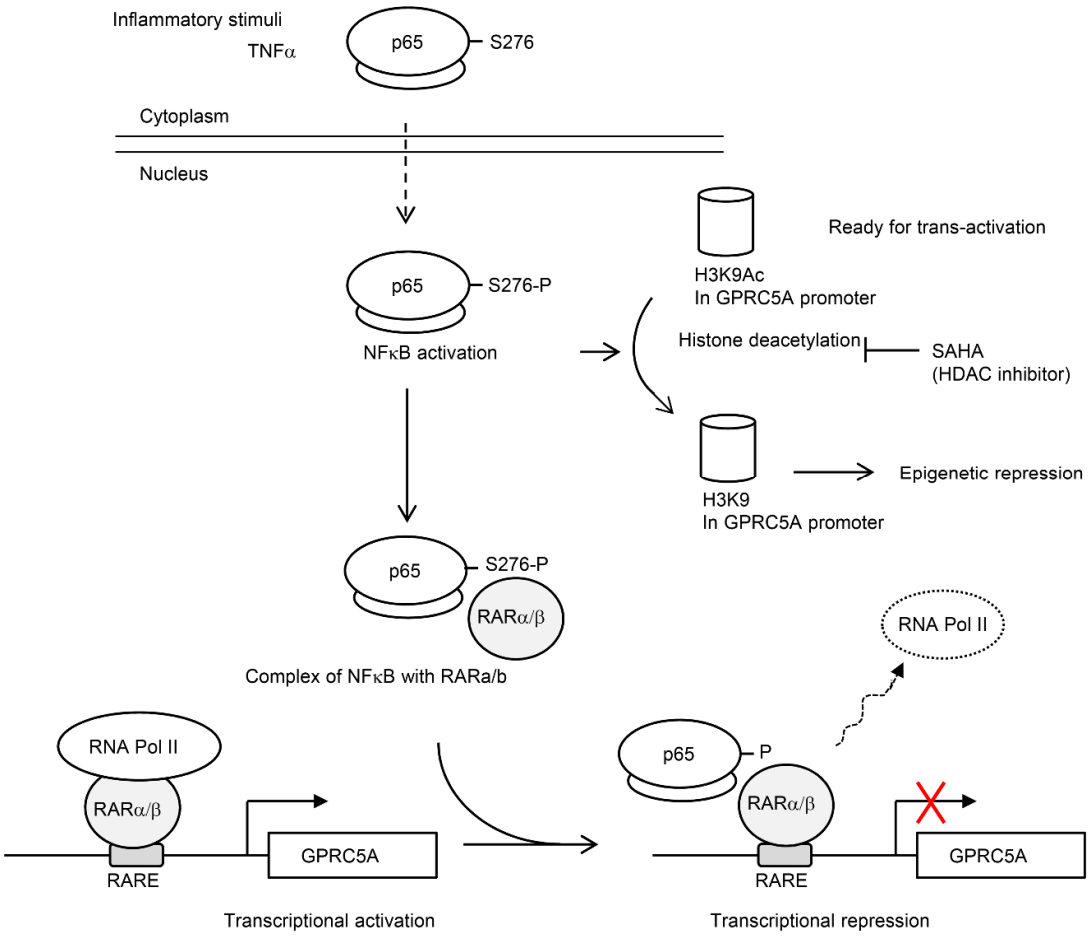


Figure 8. The proposed model of NF-κB-mediated repression of GPRC5A in lung epithelial cells.