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Sin3B mediates collagen type I gene repression by interferon gamma in vascular smooth muscle cells

Xinyu Weng [1], Xian Cheng [1], Xiaoyan Wu, Huihui Xu, Mingming Fang, Yong Xu *

Atherosclerosis Research Center, Key Laboratory of Cardiovascular Disease and Molecular Intervention, Department of Pathophysiology, Nanjing Medical University, China

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ABSTRACT

Collagen type I is the primary component of the extracellular matrix (ECM). Repression of collagen type I gene (*COL1A2*) transcription by the pro-inflammatory cytokine interferon gamma (IFN-γ) in vascular smooth muscle cells (VSMCs) is a key step during atherogenesis that leads to the destabilization of the atherosclerotic plaque. The epigenetic mechanism underlying IFN-γ induced *COL1A2* repression is not clearly appreciated. We show here that Sin3B, a component of the eukaryotic histone deacetylase (HDAC) complex, was recruited to *COL1A2* transcription start site in response to IFN-γ treatment in VSMCs paralleling *COL1A2* repression. Short hairpin RNA (shRNA) mediated silencing of Sin3B abrogated collagen repression by IFN-γ and blocked the erasure of active histone marks and the accumulation of repressive histone marks on *COL1A2* transcription start site as evidenced by chromatin immunoprecipitation (ChIP) assays. Sin3B cooperated with G9a, a histone H3K9 methyltransferase, to induce a repressive chromatin structure surrounding the collagen gene transcription start site in response to IFN-γ stimulation. Sin3B was recruited by regulatory factor for X-box 5 (RFX5) to the collagen site through a mechanism that involved HDAC2 mediated deacetylation of RFX5. Together, our data indicate that a repressor complex that contains RFX5, HDAC2, Sin3B, and G9a is responsible for IFN-γ induced *COL1A2* repression in VSMCs. Targeting individual component of this complex will likely yield potential therapeutic solutions against atherosclerosis.

1. Introduction

Collagen type I is the most abundant protein in the extracellular matrix (ECM). Expression of the collagen type I gene (*COL1A2*) takes place primarily at the transcriptional level and is intimately related to the pathogenesis of a host of human diseases including atherosclerosis [1]. Atherosclerosis is a multifaceted pathologic process that involves the interplay between different cells within the vasculature [2]. For instance, T lymphocytes attracted to the atherosclerotic lesions can secret several pro-inflammatory cytokines including interferon gamma (IFN-γ) [3]. IFN-γ, in turn, acts on vascular smooth muscle cells (VSMCs), the major source of collagen type I synthesis in the atherosclerotic plaque, to down-regulate *COL1A2* transcription. *COL1A2* repression leads to the thinning of the fibrous cap and eventually its rupture, which causes a series of complications contributing to increased mortality of patients with atherosclerosis [4].

Investigations in the past decade have uncovered several pathways that mediate IFN-γ induced *COL1A2* transcriptional repression. Higashi et al. have found that Y box binding protein 1 (YB-1) binds to the proximal *COL1A2* (−161/−150) promoter and represses *COL1A2* transcription possibly by blocking the access of p300 [5,6]. Varga and colleagues suggest that CCAAT/enhancer-binding protein b (C/EBPβ) is necessary for IFN-γ induced *COL1A2* in human neonatal foreskin fibroblasts by binding to a more distal site (−353/−186). We on the other hand have previously discovered a binding motif near the *COL1A2* gene transcriptional start site (−30/+25) for regulatory factor for X-box (RFX) family of transcription factors [7–9]. IFN-γ promotes the binding of RFX5 on the *COL1A2* transcription start site in human lung fibroblast cells; once bound RFX5 represses *COL1A2* transcription by recruiting a histone deacetylase (HDAC) complex that includes HDAC2 and Sin3B.

Initially identified as a repressor of the *HO* gene in *Saccharomyces cerevisiae*, Sin3B is conserved in all major eukaryotic organisms [10]. Sin3B is usually found in large mega-protein complexes that include histone modifying enzymes and chromatin remodelers [11]. Despite rigorous research, several outstanding questions remain unanswered regarding the role of Sin3B in *COL1A2* trans-repression by IFN-γ. Is Sin3B absolutely required for IFN-γ induced

* Corresponding author. Address: Nanjing Medical University, Nanjing, Jiangsu 210029, China. Fax: +86 25 86862888.
E-mail address: yjxu@njmu.edu.cn (Y. Xu).
[1] These authors contributed equally to this work.

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COL1A2 repression in VSMCs? If so, what is the underlying potential epigenetic mechanism(s)? Is there a crosstalk between Sin3B and other epigenetic factors? How is Sin3B recruited to *COL1A2* promoter? We present evidence in this report that Sin3B, recruited by RFX5 in a deacetylation-dependent manner, is essential for IFN-γ induced *COL1A2* repression in VSMCs by cooperating with G9a to maintain a repressive chromatin structure surrounding the collagen gene transcription start site.

2. Methods and materials

2.1. Cell culture and treatment

Rat vascular smooth muscle cell (A10) and human embryonic kidney cell (HEK293) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone). Human aortic smooth muscle cell (HASMC) was purchased from Lonza and maintained in SMBM with supplements supplied by the vendor. Human and rat recombinant IFN-γ were from R&D.

2.2. Plasmids, transient transfection, viral infection, and luciferase assay

FLAG-tagged RFX5, FLAG-tagged HDAC1-3, Myc-tagged Sin3B, and *col1a2* promoter luciferase construct (pH20) have been described previously [12–14]. shRNA plasmid targeting RFX5, Sin3B, G9a, and control shRNA plasmid were purchased from Sigma. Lentiviral particles were generated as previously described [15].

2.3. Protein extraction, immunoprecipitation and Western

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer with freshly added protease inhibitor (Roche). Antibodies were incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads. Alternatively, FLAG-conjugated beads were incubated with lysates overnight. Precipitated immune complex was eluted with 3X FLAG peptide (Sigma). Western blotting analyses were performed with anti-FLAG, anti-β-actin (Sigma), anti-collagen type I, anti-RFX5 (Rockland), anti-acetyl lysine (Cell Signaling), anti-G9a (Upstate), and anti-Sin3B (Santa Cruz) antibodies.

2.4. Chromatin immunoprecipitation (ChIP) and Re-ChIP

ChIP and Re-ChIP assays were performed essentially as described previously [16,17]. Chromatin was cross-linked with 1% formaldehyde. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-RFX5 (Rockland), anti-HDAC2, anti-Sin3B (Santa Cruz), anti-G9a, anti-acetyl histone H3, anti-acetyl histone H4, anti-trimethylated histone H3K4, and anti-dimethylated H3K9 (Upstate). For Re-ChIP, immune complexes were diluted with the re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris pH 8.1), and subject to immunoprecipitation with a second antibody of interest. Precipitated genomic DNA was amplified by real-time PCR with primers as previously described [13].

2.5. RNA extraction and real-time PCR

RNA was extracted using an RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a Super-Script First-strand synthesis system (Invitrogen). Real-time PCR reactions were performed on an ABI STEPONE Plus (Life Tech) with previously described primers [9,18].

2.6. In vitro histone deacetylase (HDAC) assay

HDAC assay was performed with a commercially available kit (Upstate). Briefly, immune complex that might contain an HDAC activity was incubated with [^{3}H]-acetyl-Histone H4 peptide in HDAC assay buffer in a 37 °C water bath overnight. Released [^{3}H]-acetate was measured using a scintillation counter.

2.7. Statistical analysis

One-way ANOVA with post hoc Scheffe analyses were performed using an SPSS package. *P* values smaller than .05 were considered statistically significant.

3. Results

3.1. Sin3B is essential for IFN-γ induced COL1A2 repression in smooth muscle cells

We have previously demonstrated that Sin3B might be involved in transcriptional repression of collagen type I gene (*COL1A2*) by IFN-γ in human lung fibroblast cells [14,19]. In order to determine whether Sin3B is indispensible for IFN-γ induced *COL1A2* repression in smooth muscle cells, the following experiments were performed. When human primary aortic smooth muscle cells (HASMCs) were treated with IFN-γ, *COL1A2* message levels were down-regulated in a time course-dependent manner: *COL1A2* mRNA was repressed by ~60% after 48 h of treatment (Fig. 1A). When ChIP assays were performed, we found that Sin3B binding on the collagen gene transcription start site was up-regulated with time paralleling the down-regulation of *COL1A2* messages. In the meantime, active histone marks including acetylated histone H3 (AcH3), acetylated histone H4 (AcH4), and trimethylated histone H3 lysine 4 (H3K4Me3) started to disappear from the collagen gene transcription start site whereas dimethylated histone H3 lysine 9 (H3K9Me2), which predicts transcriptional repression, started to accumulate.

Next, we transfected rat vascular smooth muscle cells (A10) with a *Col1a2* promoter-luciferase construct with increasing doses of Sin3B. As shown in Fig. 1B, Sin3B enhanced the repression of *Col1a2* promoter activity by IFN-γ in a dose-dependent manner. In contrast, when endogenous Sin3B was silenced with a short hairpin RNA (shRNA) plasmid (shSin3B), repression of the *col1a2* promoter by IFN-γ was abrogated (Fig. 1C). Finally, we infected HASMCs with lentivirus carrying shRNA targeting either Sin3B or LacZ (shLacZ) followed by treatment with IFN-γ. In the absence of Sin3B, IFN-γ failed to repress mRNA (Fig. 1D) or protein (Fig. 1E) levels of collagen type I. Together these results suggest that Sin3B is recruited to the collagen gene transcription start site following IFN-γ stimulation and is required for *COL1A2* transcriptional repression in smooth muscle cells.

3.2. Sin3B maintains a repressive chromatin structure surrounding COL1A2 transcription start site

Sin3B regulates transcription by influencing the chromatin structure [20]. Since ChIP assay showed that occupancy of Sin3B on the collagen gene transcription start site following IFN-γ treatment coincided with the disappearance of active histone marks (AcH3, AcH4, and H3K4Me3) and acquisition of repressive histone modification (H3K9Me2, Fig. 1A) at the same site, we set out to evaluate the role of Sin3B in the maintenance of a repressive chromatin structure surrounding *COL1A2* transcription start site. To this end, HASMCs were infected with lentivirus carrying either shSin3B or shLacZ; infection with shSin3B-carrying lentivirus

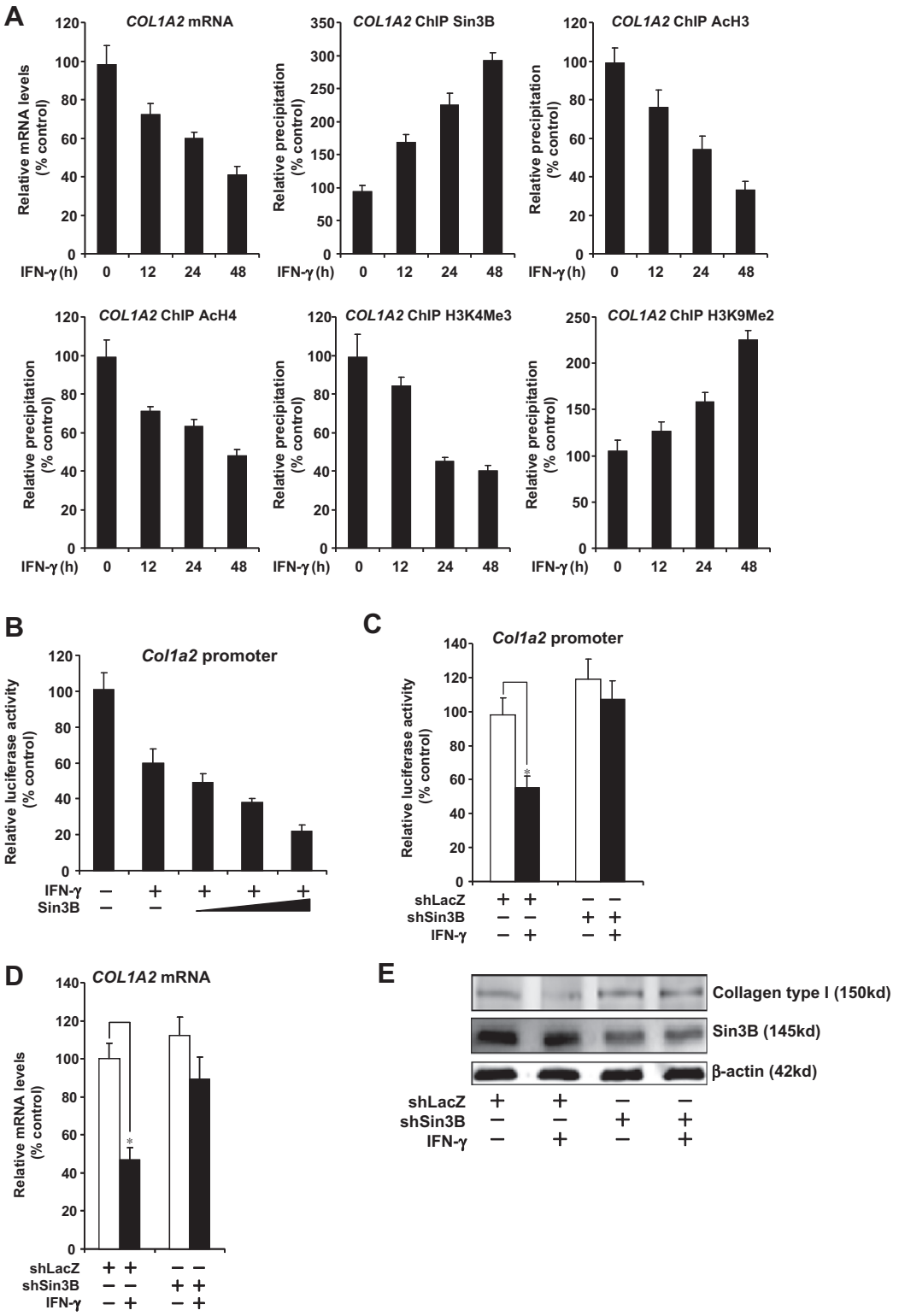

Fig. 1. Sin3B is essential for IFN-γ induced *COL1A2* repression in smooth muscle cells. (A) HASMCs were treated with IFN-γ (100 U/ml) and harvested at indicated time points. Collagen type I (*COL1A2*) mRNA levels were measured by qPCR. Accumulation of Sin3B and histones was evaluated by ChIP. Precipitated DNA was amplified using primers surrounding *COL1A2* transcription start site. (B) A collagen promoter construct was transfected into A10 cells with or without Sin3B followed by treatment with IFN-γ. Luciferase activities were normalized by both protein concentration and GFP fluorescence. (C) A collagen promoter construct was transfected into A10 cells with shRNA plasmid targeting Sin3B (shSin3B) or LacZ (shLacZ) followed by treatment with IFN-γ. Luciferase activities were normalized by both protein concentration and GFP fluorescence. (D and E) HASMCs were infected with lentiviral particles carrying shRNA targeting either Sin3B or LacZ followed by treatment with IFN-γ. mRNA (D) and protein (E) levels of collagen type I were examined by qPCR and Western blotting, respectively.

completely prevented the recruitment of Sin3B by IFN-γ stimulation compared to the shLacZ-infected cells (Fig. 2A). Sin3B knockdown restored active histone marks including AcH3 (Fig. 2B), AcH4 (Fig. 2C), and H3K4Me3 (Fig. 2D) while prevented the accumulation of H3K9Me2 (Fig. 2E) on the collagen gene transcription start site in IFN-γ treated HASMCs. Thus, Sin3B indeed is

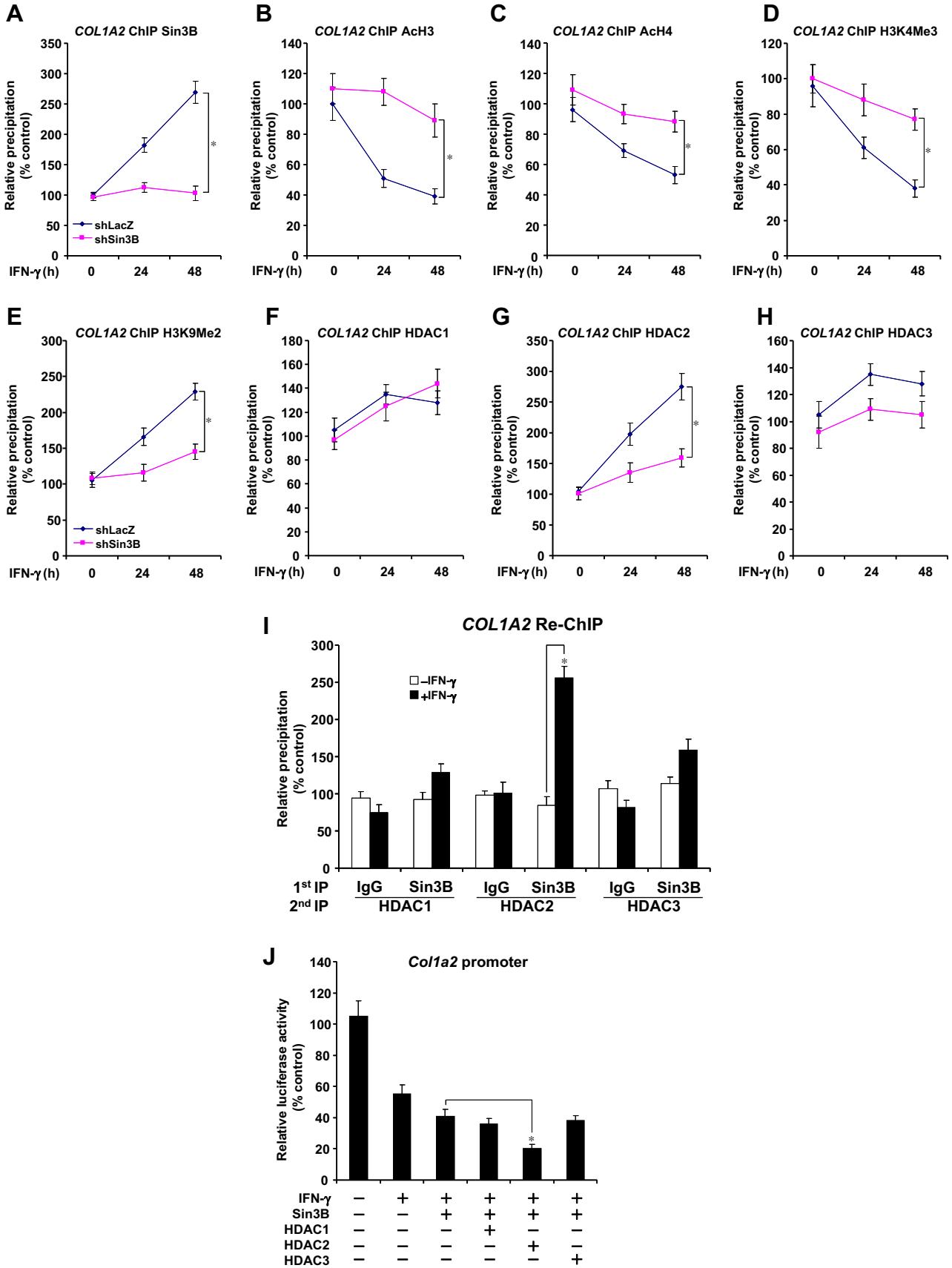

Fig. 2. Sin3B is essential for the maintenance of a repressive chromatin structure surrounding the collagen gene transcription start site. (A–H) HASMCs were infected with lentiviral particles carrying shRNA targeting either Sin3B or LacZ followed by treatment with IFN-γ. ChIP assays were performed with anti-acetyl Sin3B (A), anti-acetyl H3 (B), anti-acetyl H4 (C), anti-trimethyl H3K4 (D), anti-dimethyl H3K9 (E), anti-HDAC1 (F), anti-HDAC2 (G), or anti-HDAC3 (H). (I) HASMCs were treated with or without IFN-γ for 48 h. Re-ChIP assay were performed with indicated antibodies. (J) A collagen promoter construct was transfected into A10 cells with indicated expression constructs followed by treatment with IFN-γ.

responsible for the establishment of a repressive chromatin structure surrounding *COL1A2* transcription start site in response to IFN-γ stimulation.

Sin3B is usually found a large complex with various class I histone deacetylases (HDACs) [21]. Interestingly, HDAC2 (Fig. 2G), but not HDAC1 (Fig. 2F) or HDAC3 (Fig. 2H), was recruited to *COL1A2* transcription start site in response to IFN-γ stimulation in a similar kinetics as Sin3B. More importantly, HDAC2 binding was disrupted without Sin3B (Fig. 2G). In addition, Re-ChIP assay also revealed a Sin3B-HDAC2 complex assembled on the collagen gene transcription start site in HASMCs treated with IFN-γ (Fig. 2I). Finally, reporter assay showed that co-expression of HDAC2, but not HDAC1 or HDAC3, resulted in further repression of the collagen promoter activity in the presence of Sin3B (Fig. 2J). Collectively, these data suggest that Sin3B binding on *COL1A2* transcription start site renders the surrounding chromatin structure repressive presumably by recruiting HDAC2.

3.3. Sin3B interacts with G9a to repress collagen type I transcription in smooth muscle cells

Since we observed that Sin3B knockdown prevented the accumulation of H3K9Me2, catalyzed by the histone methyltransferase G9a [22], on the collagen gene transcription start site, we hypothesized that Sin3B might interact with G9a and engage G9a in collagen gene repression. Reciprocal immunoprecipitation clearly showed that anti-Sin3B co-precipitated G9a in HASMCs and vice versa (Fig. 3A). Furthermore, IFN-γ treatment increased the occupancy of G9a (Fig. 3B) and stimulated the formation of a Sin3B–G9a complex (Fig. 3C) on the collagen gene transcription start site as evidenced by ChIP and Re-ChIP assays, respectively. Co-expression of G9a and Sin3B down-regulated the collagen promoter activity more potently than the expression of either protein individually did (Fig. 3D). On the contrary, shRNA-mediated silencing of G9a blocked the repression of COL1A2 expression by IFN-g at both message (Fig. 3E) and protein (Fig. 3F) levels. Therefore, G9a, similar to Sin3B, might also be an integral part of the pathway whereby IFN-γ represses the transcription of *COL1A2* gene.

Next, we evaluated the role of G9a in modulating the chromatin structure surrounding *COL1A2* transcription start site. Silencing of G9a by shRNA decreased the binding of G9a and erased the accumulation of H3K9Me2 on the collagen gene transcription start site as expected (Fig. 3G and H). Of interest, G9a ablation also partially restored the levels of AcH3 (Fig. 3I), AcH4 (Fig. 3J), and H3K4Me3 (Fig. 3K) on the collagen gene transcription start site, although this effect was more modest when compared to Sin3B silencing (Fig. 2B–D). These data suggest that Sin3B might contribute to IFN-γ induced *COL1A2* repression in part by enlisting G9a to influence the chromatin structure surrounding *COL1A2* transcription start site.

3.4. RFX5 recruits Sin3B to the collagen gene transcription start site in a deacetylation-dependent manner

Sin3B by itself has no preferential affinity for any particular stretch of DNA sequence; instead, it relies on sequence-specific transcription factors to be recruited to the chromatin to participate in transcriptional regulation [23]. Previously we have shown by DNA affinity pull-down assay that regulatory factor for X box 5 (RFX5) might be required for Sin3B recruitment to the collagen gene transcription start site in lung fibroblast cells [19]. Indeed, Re-ChIP data confirmed that a Sin3B–RFX5 was clearly detectable upon IFN-γ stimulation on the collagen gene transcription start site in HASMCs (Fig. 4A). Moreover, *in vitro* HDAC assay showed that RFX5 was associated with an HDAC activity in HASMCs and that IFN-γ treatment increased that activity (Fig. 4B), indicative a stronger Sin3B–RFX5 interaction. On the other hand, silencing of RFX5 by shRNA blocked the binding of both RFX5 (Fig. 4C) and Sin3B (Fig. 4D), supporting an essential role for RFX5 in recruiting Sin3B to the collagen site. In addition, RFX5 knockdown also blocked the binding of G9a (Fig. 4E) and normalized the levels of AcH3 (Fig. 4F), AcH4 (Fig. 4G), H3K4Me3 (Fig. 4H), and H3K9Me2 (Fig. 4I) on the collagen gene transcription start site. These data strongly indicate that the ability of RFX5 to mediate IFN-γ induced *COL1A2* repression is likely a function of its interaction with epigenetic modulators such as Sin3B.

RFX5 protein is acetylated *in vivo* [13,19]. Over-expression of wild type (WT) HDAC2, but not two enzyme deficient (M1, M2) mutants of HDAC2, in HEK293 cells significantly down-regulated RFX5 acetylation (Fig. 4J). IFN-γ treatment led to a stronger interaction between RFX5 and HDAC2 on *COL1A2* transcription start site and decreased RFX5 acetylation in HASMCs (Fig. 4K). Intriguingly, deacetylation of RFX5 by HDAC2 promoted its interaction with Sin3B (Fig. 4L). More importantly, HDAC2-mediated RFX5 deacetylation enhanced Sin3B recruitment to the collagen gene transcription start site in response to IFN-γ treatment (Fig. 4M). Therefore, HDAC2-mediated deacetylation of RFX5 serves as a pre-requisite for Sin3B recruitment and *COL1A2* repression.

4. Discussion

COL1A2 repression by IFN-γ in VSMCs constitutes a key step towards the destabilization and rupture of the atherosclerotic plaque. In the current report we demonstrate a novel epigenetic mechanism underlying this process (Fig. 4N): in response to IFN-γ stimulation, RFX5 recruits HDAC2 and becomes deacetylated. Once deacetylated RFX5 then recruits Sin3B to the collagen site, which interacts with G9a and HDAC2 to remove histone H3 and H4 acetylation and augment H3K9 dimethylation establishing a repressive chromatin structure. This chain of events eventually leads to *COL1A2* transcriptional down-regulation.

Previous reports have unequivocally linked the function of Sin3B to its ability to interact with class I HDACs [21]. We confirm here that HDAC2 instead of HDAC1 or HDAC3 was recruited by Sin3B to *COL1A2* transcription start site to erase acetylated histones H3 and H4. More importantly, we show here that G9a, a histone H3K9 methyltransferase G9a, was also in a complex with Sin3B to repress *COL1A2* transcription. Sin3B possess several amphipathic helix-loop-helix motifs that are required for its interaction with other epigenetic factors and/or transcription factors whereas G9a is known to interact with proteins via such a structure [24–26]. Therefore, we suspect Sin3B might bring G9a to the collagen transcription start site through this interface to up-regulate H3K9Me2 levels as part of a scheme to repress *COL1A2* expression. Alternatively, Sin3B has been shown to down-regulate acetylation of H3K9, a modification incompatible with H3K9Me2 [27]. Thus, Sin3B could directly up-regulate H3K9Me2 following H3K9 deacetylation independent of G9a, which might explain the observation that G9a silencing had a more moderate effect than Sin3B (Fig. 2). Down-regulation of AcH3 and AcH4 and up-regulation of H3K9Me2 by the Sin3B–HDAC2–G9a complex might conceivably suppress H3K4Me3 levels since these modifications are mutually exclusive. At this point, however, other possibilities cannot be entirely excluded. For instance, Sin3B can found on H3K4Me-enriched nucleosomes [28]. It is likely that Sin3B might actively remove H3K4Me3 by recruiting a demethylase such as the CoREST/LSD1 complex [29]. Additional investigation is warranted to clarify the role of Sin3B in modulating histone modifications in the process of IFN-γ induced *COL1A2* repression and beyond.

Another major finding of this report is that HDAC2-mediated RFX5 appears to be indispensible for Sin3B recruitment. Unlike our previous finding that deacetylation of RFX5 by class III deacetylase SIRT1 targets RFX5 for proteasomal degradation [13],

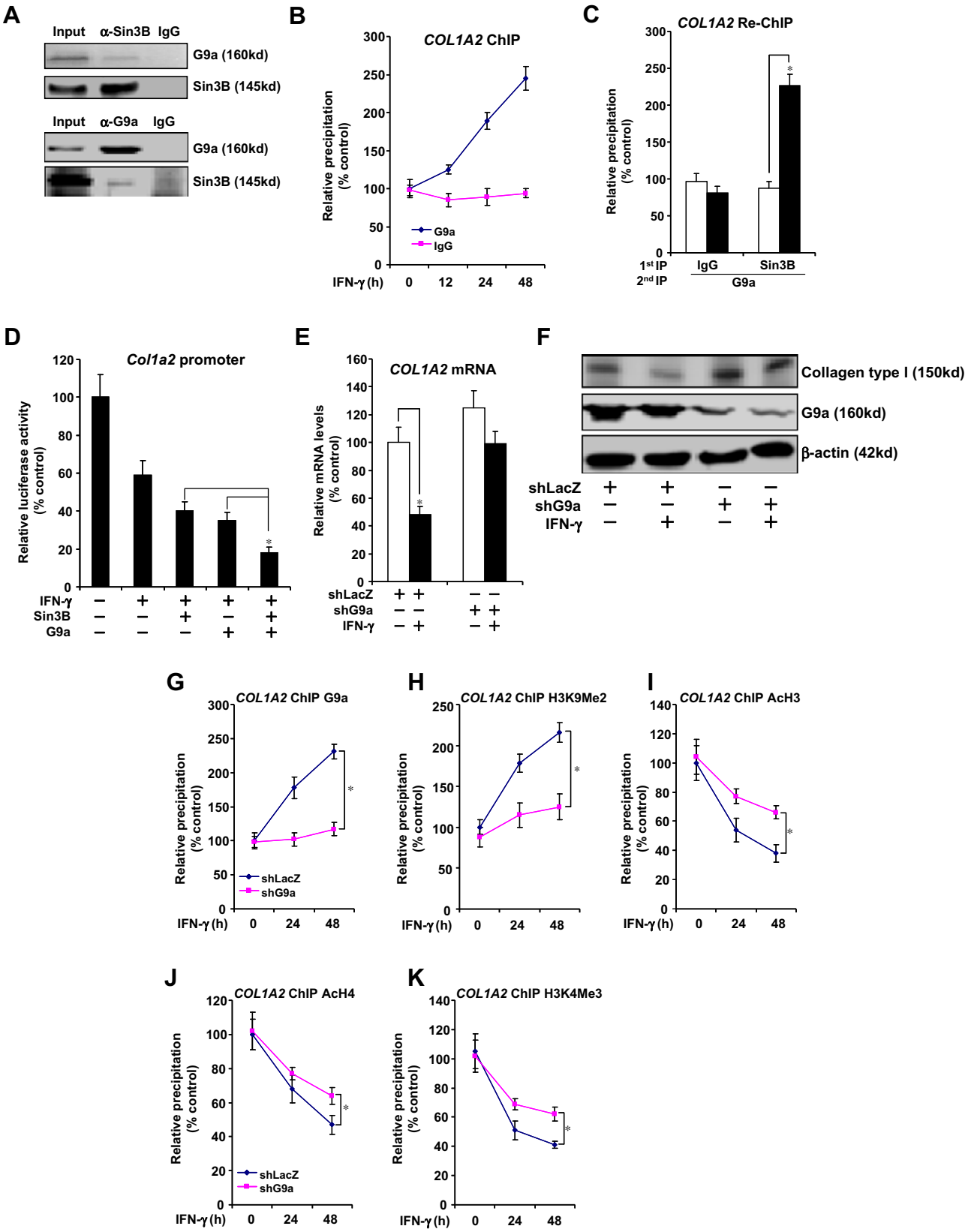

Fig. 3. Sin3B interacts with G9a to repress collagen type I transcription in smooth muscle cells. (A) Immunoprecipitation assays were performed using HASMC whole cell lysates with indicated antibodies. 10% of the starting material was loaded as input. (B) HASMCs were treated with IFN-γ (100 U/ml) and harvested at indicated time points. ChIP assays were performed with anti-G9a or IgG. Precipitated DNA was amplified using primers surrounding *COL1A2* transcription start site. (C) HASMCs were treated with or without IFN-γ for 48 h. Re-ChIP assay were performed with indicated antibodies. (D) A collagen promoter construct was transfected into A10 cells with indicated expression constructs followed by treatment with IFN-γ. (E and F) HASMCs were infected with lentiviral particles carrying shRNA targeting either G9a or LacZ followed by treatment with IFN-γ. mRNA (E) and protein (F) levels of collagen type I were examined by qPCR and Western blotting, respectively. (G–K) HASMCs were infected with lentiviral particles carrying shRNA targeting either G9a or LacZ followed by treatment with IFN-γ. ChIP assays were performed with anti-G9a (G), anti-dimethyl H3K9 (H), anti-acetyl H3 (I), anti-acetyl H4 (J), and anti-trimethyl H3K4 (K).

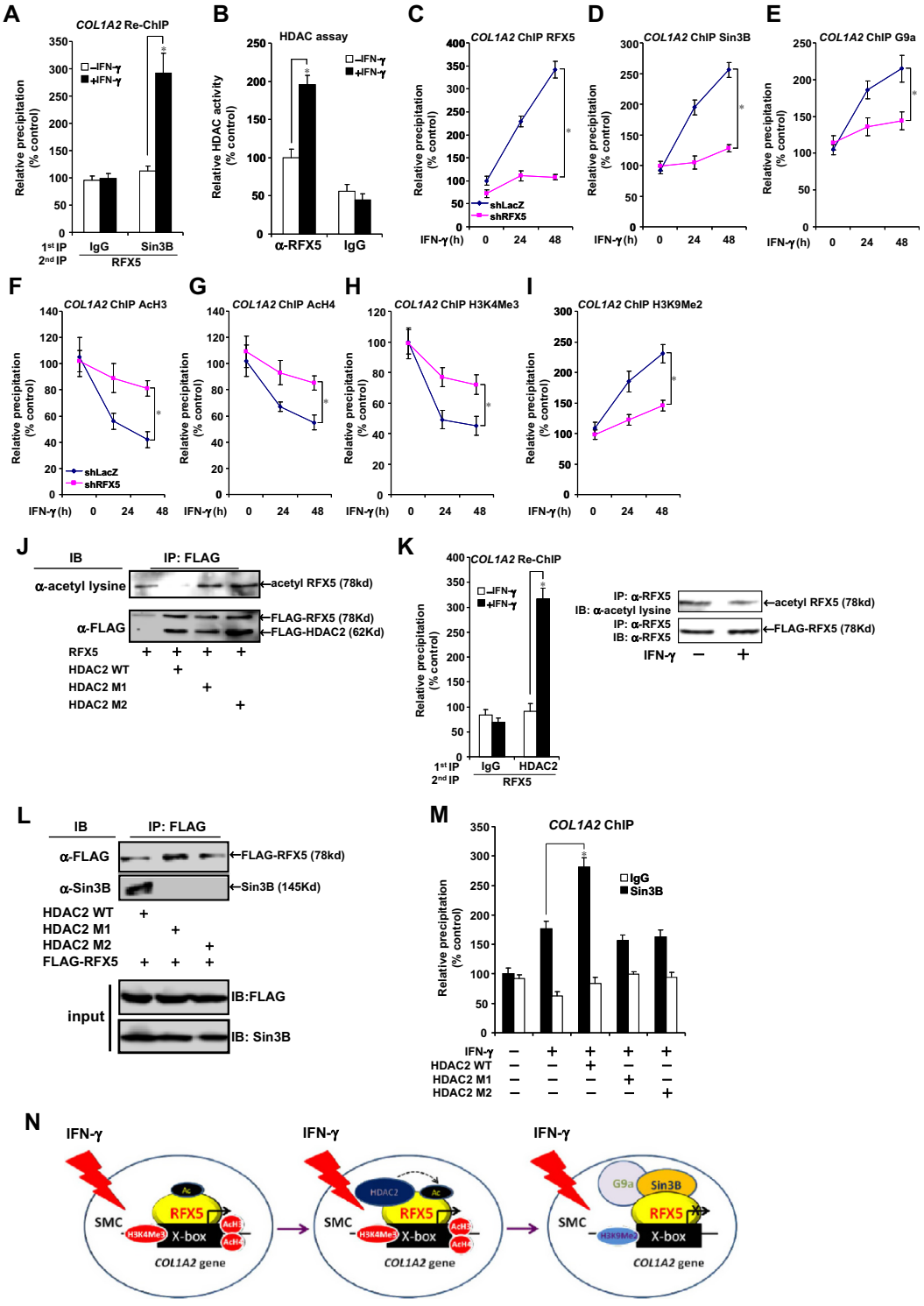

Fig. 4. RFX5 recruits Sin3B to the collagen gene transcription start site in a deacetylation-dependent manner. (A) HASMCs were treated with or without IFN-γ for 48 h. Re-ChIP assay were performed with indicated antibodies. Precipitated DNA was amplified using primers surrounding *COL1A2* transcription start site. (B) HASMCs were treated with or without IFN-γ for 48 h. Whole cell lysates were immunoprecipitated with anti-RFX5 or IgG and the precipitated immune complex was subject to *in vitro* HDAC assay as described under Section 2. (C–I) HASMCs were infected with lentiviral particles carrying shRNA targeting either RFX5 or LacZ followed by treatment with IFN-γ. ChIP assays were performed with anti-RFX5 (C), anti-Sin3B (D), anti-G9a (E), anti-acetyl H3 (F), anti-acetyl H4 (G), anti-dimethyl H3K9 (H), and anti-trimethyl H3K4 (I). (J) HEK293 cells were transfected with FLAG-tagged RFX5 and either wild type (WT) or mutated (M1, M2) HDAC2. Immunoprecipitation was performed with anti-FLAG and acetylation of RFX5 was assessed by Western blotting with anti-acetyl lysine. (K) HASMCs were treated with or without IFN-γ for 48 h. The cells were divided into two fractions. (Left) One half was used to perform Re-ChIP assay with indicated antibodies. (Right) The other half was used for immunoprecipitation with anti-RFX5. Acetylation levels of RFX5 were probed by Western blotting. (L) HEK293 cells were transfected with FLAG-tagged RFX5 and either wild type (WT) or mutated (M1, M2) HDAC2. Immunoprecipitation was performed with anti-FLAG and Western blotting analysis was performed with anti-FLAG or anti-Sin3B. (M) HEK293 cells were transfected with either wild type (WT) or mutated (M1, M2) HDAC2 followed by treatment with IFN-γ for 48 h. ChIP assays were performed with anti-Sin3B or IgG. (N) A model depicting the mechanism of *COL1A2* repression by IFN-γ in VSMCs.

HDAC2 did not alter RFX5 protein stability; instead, HDAC2 promotes the interaction between RFX5 and Sin3B. This creates a positive feedback loop: Sin3B is necessary for HDAC2 binding on *COL1A2* promoter (Fig. 2) whereas HDAC2, by its virtue of deacetylating RFX5, may bring additional Sin3B perpetuating and stabilizing the formation of a repressor complex. This model does not rule out the possibility that Sin3B could be directly modified by HDAC2, which may change its conformation to support a stronger interaction with RFX5. It has been shown that Sin3B can be ubiquitinated *in vivo* [30]. It would be of great importance to determine whether Sin3B could undergo other post-translational modifications (PTM) in a stimuli-dependent manner (e.g., IFN-γ) and how these PTMs may influence Sin3B function.

In summary, we highlight an essential role for Sin3B in IFN-γ induced *COL1A2* repression in smooth muscle cells. Sin3B exerts a wide range of effects on cellular functions that are relevant to atherogenesis [31]. Future studies using tissue-specific Sin3B transgenic animal model will further benefit the effort of exploiting the current dataset in the development of novel anti-atherosclerosis therapeutics.

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