ORIGINAL RESEARCH

Coactivator-Associated Arginine Methyltransferase-1 Function in Alveolar Epithelial Senescence and Elastase-Induced Emphysema Susceptibility

Rim S. J. Sarker[1], Gerrit John-Schuster[1], Alexander Bohla[1], Kathrin Mutze[1], Gerald Burgstaller[1], Mark T. Bedford[2], Melanie Königshoff[1], Oliver Eickelberg[1,3], and Ali Ö. Yildirim[1]

[1]Comprehensive Pneumology Center, Institute of Lung Biology and Disease, Helmholtz Zentrum München, Member of the German Center for Lung Research, Neuherberg, Germany; [2]Department of Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, Smithville, Texas; and [3]University Hospital of the Ludwig-Maximilians-University, München, Germany

Abstract

Chronic obstructive pulmonary disease (COPD) is characterized by an irreversible loss of lung function and is one of the most prevalent and severe diseases worldwide. A major feature of COPD is emphysema, which is the progressive loss of alveolar tissue. Coactivator-associated arginine methyltransferase-1 (CARM1) regulates histone methylation and the transcription of genes involved in senescence, proliferation, and differentiation. Complete loss of CARM1 leads to disrupted differentiation and maturation of alveolar epithelial type II (ATII) cells. We thus hypothesized that CARM1 regulates the development and progression of emphysema. To address this, we investigated the contribution of CARM1 to alveolar rarefication using the mouse model of elastase-induced emphysema *in vivo* and small interfering (si)RNA-mediated knockdown in ATII-like LA4 cells *in vitro*. We demonstrate that emphysema progression *in vivo* is associated with a time-dependent down-regulation of CARM1. Importantly, elastase-treated CARM1 haploinsufficient mice show significantly increased airspace enlargement (52.5 ± 9.6 μm versus 38.8 ± 5.5 μm; $P < 0.01$) and lung compliance (2.8 ± 0.32 μl/cm H_2O versus 2.4 ± 0.4 μl/cm H_2O; $P < 0.04$) compared with controls. The knockdown of CARM1 in LA4 cells led to decreased sirtuin 1 expression (0.034 ± 0.003 versus 0.022 ± 0.001; $P < 0.05$) but increased expression of p16 (0.27 ± 0.013 versus 0.31 ± 0.010; $P < 0.5$) and p21 (0.81 ± 0.088 versus 1.28 ± 0.063; $P < 0.01$) and higher β-galactosidase–positive senescent cells ($50.57 \pm 7.36\%$ versus $2.21 \pm 0.34\%$; $P < 0.001$) compared with scrambled siRNA. We further demonstrated that CARM1 haploinsufficiency impairs transdifferentiation and wound healing ($32.18 \pm 0.9512\%$ versus $8.769 \pm 1.967\%$; $P < 0.001$) of alveolar epithelial cells. Overall, these results reveal a novel function of CARM1 in regulating emphysema development and premature lung aging via alveolar senescence as well as impaired regeneration, repair, and differentiation of ATII cells.

Keywords: emphysema; coactivator-associated arginine methyltransferase 1; elastase-treatment; senescence; lung regeneration; alveolar type II cells

Chronic obstructive pulmonary disease (COPD) is the most common form of chronic lung disease. COPD is primarily caused by cigarette smoke (CS), air pollution, environmental factors, aging, and epigenetic modification. According to the World Health Organization COPD will stand as the third leading cause of death worldwide by the year 2030 (1). COPD is characterized by chronic bronchitis, small airway remodeling, and emphysema (2). The hallmark of emphysema is the destruction of alveolar structures leading to enlarged air spaces and reduced surface area (3). The underlying mechanism of emphysema development includes a protease–antiprotease imbalance (4), apoptosis–proliferation imbalance of epithelial and endothelial cells (5), and oxidative stress (6). More recently, COPD has been proposed to be a disease of

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Correspondence and requests for reprints should be addressed to Ali Ö. Yildirim, Ph.D., Comprehensive Pneumology Center, Institute of Lung Biology and Disease, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany. E-mail: oender.yildirim@helmholtz-muenchen.de

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Clinical Relevance

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. Emphysema, a main feature of COPD, is characterized by airspace enlargement and loss of alveolar function. Here, we show that coactivator-associated arginine methyltransferase-1 (CARM1) deficiency predisposes mice to enhanced emphysema development by regulating alveolar epithelial cell senescence and repair. This might help researchers better understand the pathogenesis of emphysema because it provides an underlying mechanism to premature senescence. The development of new pharmacological intervention regulating CARM1 activity may thus prevent emphysema development and progression. Thus, CARM1 may prove to be an effective target to treat premature lung aging in patients with emphysema.

accelerated premature aging. It has been demonstrated that emphysema development is driven by accelerated senescence of lung cells (7, 8), but the underlying mechanism of senescence is yet to be fully elucidated.

Protein arginine methyltransferases (PRMTs) are important for cellular processes, such as the regulation of senescence, cell proliferation, differentiation, and apoptosis (9, 10). The PRMT family includes 11 members classified as type I, II, or III enzymes depending on their methylation pattern (asymmetric dimethylation, symmetric dimethylation, or monomethylation, respectively) (11, 12). One member of this family is PRMT4, a type I enzyme that is also called coactivator associated arginine methyltransferase 1 (CARM1). It was originally identified as a coactivator for steroid hormone receptors (13). CARM1 methylates histone H3 and various nonhistone proteins that play essential roles in transcriptional regulation, RNA splicing, and metabolism (14, 15). Enzymatic activity of CARM1 protein is necessary for its *in vivo* functionality (16). CARM1 has been implicated in dysregulated cell proliferation of breast cancer, prostate cancer, and colorectal cancer (17–19). Importantly, the lungs of CARM1-deficient mice showed defective maturation of alveolar epithelial type II (ATII) cells and impaired transdifferentiation evident by an absence of alveolar epithelial type I (ATI) cells (20, 21). Moreover, CARM1 plays a role in regulating cellular senescence via CARM1-dependent methylation of HuR, which stabilizes SIRT1 transcripts (22, 23). HuR, an RNA binding protein, is specifically methylated by CARM1 mainly at Arg^{217} of its hinge region (24). In an animal model of elastase-induced emphysema and in a CS-induced COPD mouse model, sirtuin 1 (SIRT1) deficiency led to early development of emphysema (25). Furthermore, it has been demonstrated that there is a reduction of SIRT1 expression in the lungs of smokers and patients with COPD (26).

We hypothesized that CARM1 deficiency is involved in emphysema development by modulating cellular senescence in the lung and aimed to analyze the functional impact of the CARM1-SIRT1 axis in emphysema development. We demonstrated that CARM1 reduction was involved in the progression of elastase-induced emphysema. We showed for the first time that CARM1 heterozygous mice developed enhanced emphysema after elastase application as apparent by airspace enlargement and a decline in lung function. In addition, CARM1 reduction promoted senescence in ATII cells via a CARM1–SIRT1 axis, and CARM1 deficiency led to impaired transdifferentiation and wound healing. Taken together, our findings revealed that reduced CARM1 expression accelerates senescence of ATII cells and enhanced emphysema susceptibility.

Results from this study have partially been previously presented as an abstract at the International Conference of the American Thoracic Society 2014.

Materials and Methods

Animal Experiments

Female, 8- to 10-week–old, C57BL/6 (Charles River, Sulzfeld, Germany) and CARM1 heterozygous mice (a gift from Mark Bedford, University of Texas MD Anderson Cancer Center) were treated oropharyngeally with 80 U/kg body weight porcine pancreatic elastase (Sigma, Munich, Germany). Control mice received PBS. Wild-type (WT) mice were analyzed on Days 2, 28, 56, and 161, and CARM1 heterozygous animals were analyzed on Day 28. Experiments were repeated twice ($n = 5–6$).

Lung Function Measurement

A Biosystem XA forced maneuvers system and FinePointe RC system (Buxco, Wilmington, DE) measured forced expiratory volume in 0.1 second ($FEV_{0.1}$), forced vital capacity (FVC), functional residual capacity, dynamic compliance, and tissue elastance.

Bronchoalveolar Lavage Collection, Histology

After bronchoalveolar lavage, the right lung was snap frozen in liquid nitrogen, and the left lung was fixed at 20 cm H_2O with 6% paraformaldehyde for paraffin embedding. Details are provided in the online supplement.

Quantitative Morphometry

Mean chord length and CARM1- or SIRT1-positive stained alveolar epithelial cells were quantified using an BX51 light microscope (Olympus, Tokyo, Japan) equipped with the newCAST (Visiopharm, Hoersholm, Denmark) as described previously (27). Surfactant protein C (SP-C)-positive epithelial cells were quantified with an Axio Observer.Z1 microscope (Zeiss, Göttingen, Germany) with Axiovision 4.8 (Zeiss). The p16-positive cells were quantified by applying semiquantitative manual scoring. Details are provided in the online supplement.

Quantitative Real-Time PCR

Reverse-transcribed cDNA was amplified with Platinum SYBR Green qPCR SuperMix (Applied Biosystems, Darmstadt, Germany) on a StepOnePlus PCR System (Applied Biosystems) using HPRT1 as a reference gene. Primers are listed in Table E1 in the online supplement. Relative gene expression is presented as $2^{\Delta}CT$ ($\Delta Ct = Ct_{reference} - Ct_{target}$) and relative change to control as $2^{\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct^{control} - \Delta Ct^{treated}$).

Western Blot

Twenty micrograms of protein was separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Munich, Germany), blocked by 5% nonfat milk, and immunoblotted with anti-CARM1 (1:750; Abcam, Cambridge, UK),

anti–phospho-CARM1 (1:500; Abnova, Taipei, Taiwan), anti-SIRT1 (1:1,000; Millipore, Schwalbach, Germany), anti-p16 (1:200), anti-p21 (1:200; Santa Cruz, Heidelberg, Germany), and anti-T1α (1:4,000; R&D, Minneapolis, MN) antibodies. Upon developing with Amersham ECL Prime reagent (GE Healthcare, Freiburg, Germany), the bands were detected and quantified by Chemidoc XRS system (Bio-Rad, Munich, Germany).

Cell Culture

A murine ATII-like cell line LA-4 (ATCC, Rockville, MD) was transfected with CARM1 small interfering (si)RNAs (Qiagen, Hilden, Germany) and incubated for 48 hours. Wound healing assay was performed on transfected cells, and gap closure was determined at 0 and 16 hours using Axiovision software (Zeiss). siCARM1-transfected cells were incubated with 5% cigarette smoke extract (CSE) containing medium for 6 hours. Senescence assay was performed using a β-galactosidase staining kit (Cell Signaling, Frankfurt, Germany).

ATII Cell Isolation

Primary ATII cells were isolated from mouse lung and cultured as previously described (28). Details are provided in the online supplement.

Statistical Analysis

Mean values ± SD are given unless stated otherwise. Student's unpaired *t* test compared two groups. One-way ANOVA after Bonferroni post-test compared more than two groups if equal variances and normal distribution were given. Analyses were conducted using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Results

Single Application of Elastase Induced Progressive Pulmonary Emphysema in Mice

To investigate the underlying mechanism of emphysema development and progression, we used the porcine pancreatic elastase–induced mouse model of emphysema (27). Hematoxylin and eosin–stained lung histology confirmed a time-dependent progression of emphysema in elastase-treated mice compared with control mice (Figure 1A). As a direct measure of emphysema severity, the airspace enlargement was quantified by a quantitative morphometry of mean chord length using the newCAST system (Visiopharm). The elastase-induced airspace enlargement directly correlated with increasing dynamic lung compliance (Figure 1B). Continuous elevation in forced residual capacity (Figure 1C) and decreases of Tiffeneau index (Figure 1D) and tissue elastance (Figure 1E) until Day 161 were monitored by lung function tests and further confirmed elastase-induced emphysema progression in mice.

Coactivator-Associated Arginine Methyltransferase 1 Expression Is Diminished in Emphysematous Mouse Lungs

Coactivator-associated arginine methyltransferase 1 (CARM1) is reported to be involved in lung morphogenesis, reflected by the fact that $CARM1^{-/-}$ neonates are unable to inflate the lung with air due to an abnormal alveolar air space (20). CARM1 is also important in the regulation of proliferation and differentiation of alveolar epithelial cells during lung development (21). Based on these reports, we assessed CARM1 expression in emphysematous lungs by quantitative RT-PCR (qRT-PCR) and immunohistochemistry. We found a significant down-regulation of CARM1 at the mRNA level starting from Day 28 ($P < 0.05$) that continued to decrease until Day 161 ($P < 0.01$) compared with time-matched PBS-treated animals (Figure 2A). The involvement of CARM1 in emphysema development was further confirmed by quantification of CARM1-stained lung sections using the newCAST system, which revealed a significantly reduced percentage of CARM1-positive alveolar epithelial cells at Days 28 and 56 ($P < 0.01$). However, no differences were observed in airway epithelial cells of elastase-treated mice compared with PBS-treated animals (Figures 2B and 2C). Interestingly, we also detected that at Day 161, PBS-treated animals showed slightly reduced numbers of CARM1-positive SP-C cells, which is probably due to an aging effect in these mice. Therefore, we analyzed the levels of phosphorylated CARM1 because phosphorylation at a specific serine moiety renders CARM1 into an inactive state (29). We observed an age-dependent increase in phosphorylation of CARM1 at Day 161 in PBS-treated animals (Figures 2D and 2E). Furthermore, after elastase treatment, we detected a significant increase in phospho-CARM1 levels at Day 161 compared with Days 28 and 56. Taken together, these data suggested that emphysema progression is associated with a reduction of CARM1 expression and activity in lung alveolar epithelial cells.

Reduced CARM1 Expression in Mouse Lung Enhanced Elastase-Induced Emphysema

Because the homozygous knockout of CARM1 is lethal (20), we proceeded with CARM1 heterozygous mice to investigate its loss of function in the pathogenesis of emphysema. The level of CARM1 protein from lung homogenate was validated by Western blot (Figure 3A). Densitometric analysis confirmed that heterozygous mice had significantly reduced CARM1 at the protein level ($P < 0.01$) compared with WT mice (Figure 3B). Mean linear chord length measurement of lung histology (Figure 3C) revealed a significant airspace enlargement in CARM1 heterozygous mouse lungs compared with WT animals after elastase treatment (52.5 ± 9.6 μm versus 38.8 ± 5.5 μm; $P < 0.01$) at Day 28 (Figures 3C and 3D). No difference in airspace enlargement (22.28 ± 1.161 μm versus 23.37 ± 0.8792 μm) or lung compliance (0.001374 ± 0.00004710 μl/cm H_2O versus 0.001391 ± 0.0001266 μl/cm H_2O) was observed between WT and CARM1 heterozygous mice after PBS treatment. The higher degree of elastase-induced emphysema was also evident by a significant increase of dynamic lung compliance in CARM1 heterozygous mice compared with elastase-treated WT mice (2.8 ± 0.32 μl/cm H_2O versus 2.4 ± 0.4 μl/cm H_2O; $P < 0.05$) (Figure 3E). Our findings showed for the first time that CARM1 deficiency predisposed mice to a higher susceptibility of elastase-induced emphysema.

Reduced CARM1 Contributed to Senescence of Lung Alveolar Epithelial Cells

Previous studies reported that senescence of lung alveolar epithelial cells is involved in emphysema development (7, 8), although little is known about the causative mechanism. SIRT1 is an antisenescence gene that protects against elastase-induced emphysema via reduction of premature senescence in mice (25). Interestingly, CARM1-dependent methylated HuR is reported to stabilize the SIRT1 transcript

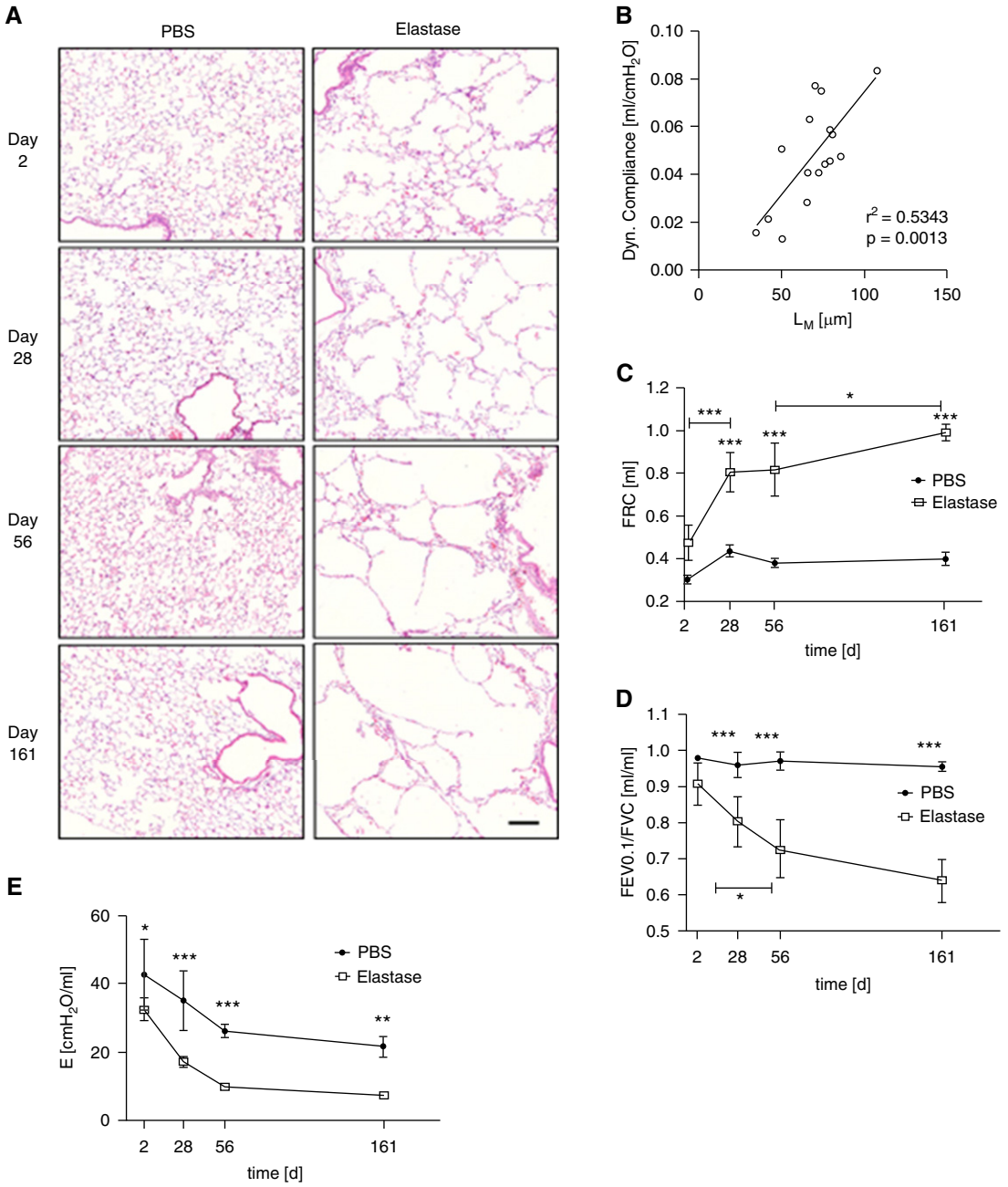

Figure 1. Analysis of elastase-induced emphysema progression. Emphysema was induced in wild-type C57BL/6 mice via oropharyngeal application of porcine pancreatic elastase of 80 U/kg body weight in 80 μl volume and analyzed on the indicated days. Mean chord length (L_M) was quantified by the stereological analysis system newCAST to determine airspace enlargement. Lung function was performed on anesthetized mice using Buxco forced maneuvers system. (*A*) Representative histological images from hematoxylin and eosin–stained lung sections. *Scale bar*: 200 μm. (*B*) Positive correlation between dynamic (Dyn.) compliance and L_M. (*C–E*) Lung function tests. (*C*) Functional residual capacity (FRC). (*D*) Tiffeneau-index. (*E*) Tissue elastance (E). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (one-way ANOVA followed by Bonferroni post-test, PBS versus elastase-treated animals). Data are presented as mean ± SD. The experiments were repeated twice ($n = 5–6$).

(22, 23). Therefore, we investigated whether reduced CARM1 could regulate alveolar epithelial cell senescence in emphysematous lungs by modulating SIRT1. Morphological analysis of SIRT1-stained sections (Figure 4A) revealed that elastase treatment significantly reduced the percentage of SIRT1-positive alveolar epithelial cells in WT animals, but it was not further decreased in CARM1 heterozygous mice (Figure 4B). In addition, we also detected fewer SIRT1-positive ATII cells in PBS-treated CARM1 heterozygous mice, indicating that CARM1 deficiency alone could regulate SIRT1 level in ATII

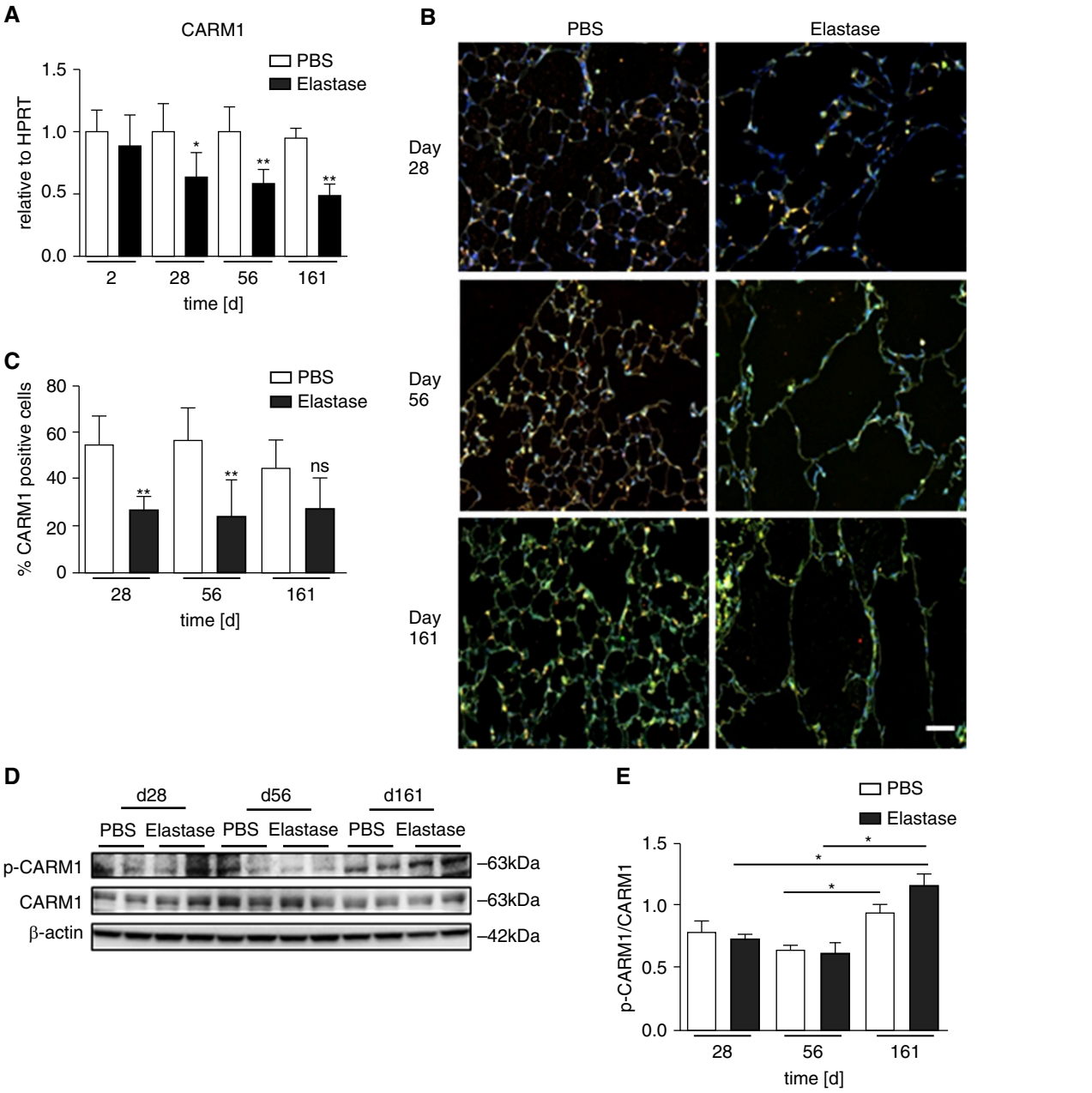

Figure 2. Down-regulation of coactivator-associated arginine methyltransferase-1 (CARM1) expression in elastase-induced emphysematous mouse lung. (*A*) CARM1 expression at the mRNA level in elastase-treated mouse lung compared with PBS-treated control animals at Days 2, 28, 56, and 161. The RNA was extracted from whole lung homogenate, and the reverse-transcribed cDNA was amplified by quantitative RT-PCR. (*B*) Representative images from immunofluorescence double staining using antibodies against CARM1 (1:150) and the alveolar epithelial type II (ATII)-specific marker surfactant protein C (SP-C) (1:100). *Green*: SP-C–positive ATII cells; *red*: CARM1-positive cells; *yellow* (merged): CARM1 and SP-C double-positive ATII cells. *Scale bar*: 10 μm. (*C*) Quantification of CARM1-positive alveolar epithelial cells by newCAST stereology system. (*D*) Representative Western blot for levels of phospho-CARM1 (p-CARM1) in lung homogenate. β-actin was used as loading control. The size of each band is indicated on the right. (*E*) Densitometric analysis of Western blot for phospho-CARM1. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test, PBS versus elastase treated animals). Data are presented as mean ± SD. The experiments were repeated twice ($n = 5$–6). HPRT, hypoxanthine-guanine phosphoribosyltransferase; ns, not significant.

cells (69.66 ± 4.55% versus 56.92 ± 1.99; $P < 0.05$). When lung homogenate was analyzed by Western blot, SIRT1 was shown to be down-regulated in emphysematous lungs. Densitometry revealed a significant reduction in SIRT1 expression in CARM1 heterozygous mice, although elastase treatment did not show further SIRT down-regulation in heterozygous mice (Figure 4C). It is likely that the SIRT1 alteration in alveolar epithelial cells is being masked by other lung cell types expressing SIRT1. Several researchers have reported that, besides stabilizing SIRT1, CARM1-dependent

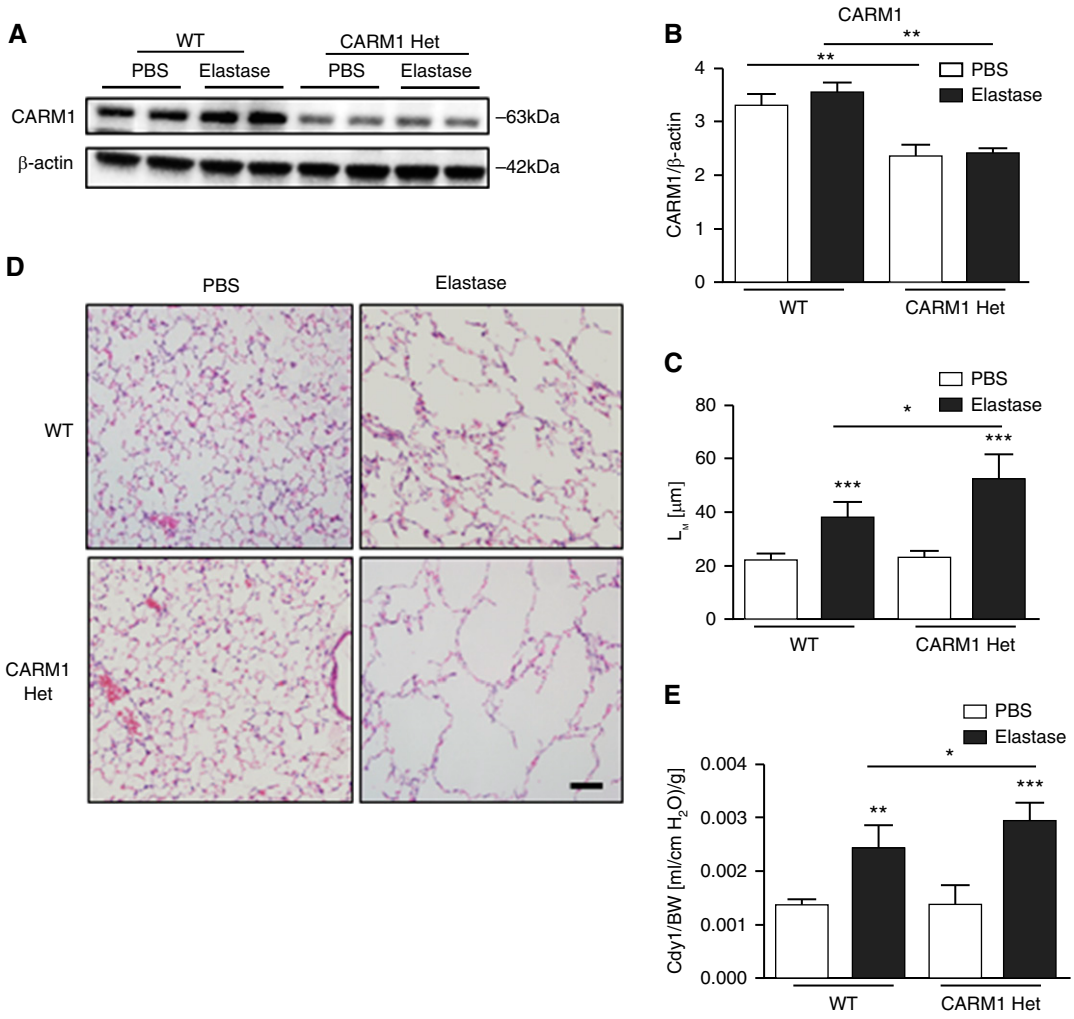

Figure 3. Enhanced airspace enlargement and increased lung function impairment in elastase-induced CARM1 heterozygous mice. Mice were analyzed on Day 28 after elastase induction. (*A*) CARM1 expression at the protein level in homogenate analyzed by Western blot. (*B*) Densitometry of the blot. $^{**}P < 0.01$ (Student's *t* test, wild-type [WT] versus CARM1 heterozygous [Het] animals). (*C*) L_M quantified by a stereological analysis system (newCAST). (*D*) Representative histological images from hematoxylin and eosin–stained lung sections. *Scale bar*: 100 μm. (*E*) Elastase-induced alterations in lung compliance normalized to the body weight (Cdy1/BW). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test versus elastase-treated CARM1 heterozygous animals). Data are presented as mean ± SD. The experiments were repeated twice ($n = 4$–7).

methylation can destabilize transcripts of p16, which is a hallmark marker for senescence (22, 30, 31). Quantifying p16-stained sections (Figure 4D) revealed that elastase treatment induced an increase in p16-positive alveolar epithelial cells in WT mice as well as in CARM1 heterozygous mice. Interestingly, control CARM1 heterozygous mice showed a significant 2.5-fold increase in basal numbers of p16-positive alveolar epithelial cells compared with WT control animals (Figure 4D). In addition, we analyzed protein expression levels of p16 and p21 in lung homogenates by Western blot. Elastase treatment increased expression of both p16 and p21 in CARM1 heterozygous mice; this change was not significant, but we could confirm that the basal p16 expression was higher in heterozygous mice compared with WT mice ($P < 0.05$) (Figure 4E). We further confirmed that PBS-treated CARM1-deficient mice showed a considerably higher number of β-galactosidase–positive alveolar epithelial cells compared with PBS-treated WT mice (Figure 4F). These results suggested that CARM1 deficiency attenuated the SIRT1-regulated antisenescence mechanism, which therefore accounted for the increased susceptibility of heterozygous mice to elastase-induced emphysema.

CARM1 Reduction Accelerated Senescence *In Vitro*

We and others previously reported (12, 21) that CARM1 is expressed by airway epithelial cells and by ATII cells in septal regions (Figure 2B). Therefore, we chose the ATII-like cell line LA-4 to study whether CARM1 could regulate senescence *in vitro*. siRNA transfection significantly reduced CARM1 expression by 70% as revealed by quantitative densitometry of Western blot (Figures 5A [*upper panel*] and 5B). Next, we analyzed protein expression of SIRT1 and noticed a significant down-regulation in siCARM1-transfected cells ($P < 0.05$) (Figures 5A [*middle panel*] and 5C).

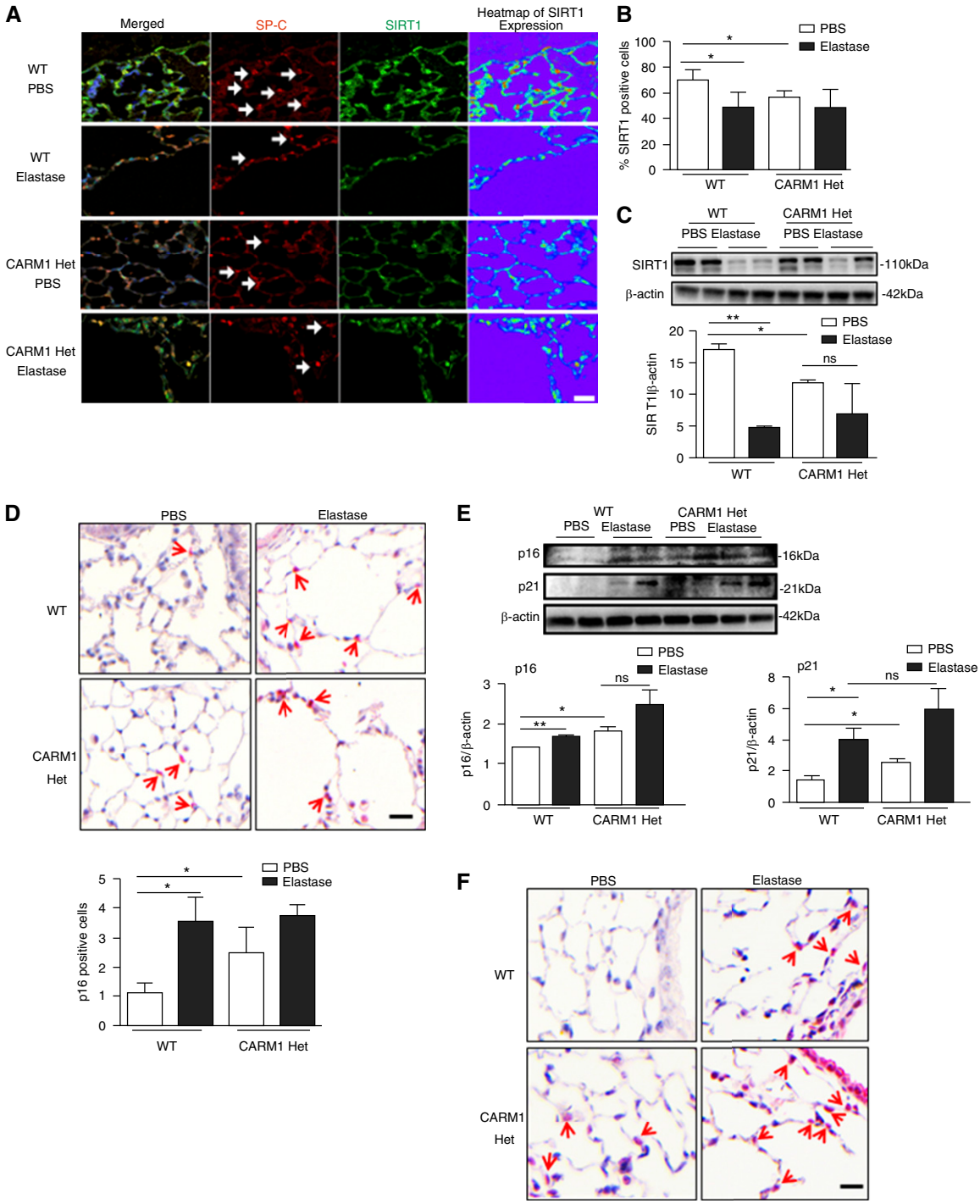

Figure 4. Loss of antisenescence SIRT1 and accumulation of senescence marker p16 in alveolar epithelial cells of CARM1 heterozygous mice. (*A*) Representative images from immunofluorescence double staining using antibodies against sirtuin 1 (SIRT1) (1:100) and SP-C, an ATII-specific marker. *Red*: SP-C–positive ATII cells (*white arrows*); *green*: SIRT1-positive cells; *yellow* (merged): SIRT1 and SP-C double-positive ATII cells. Only the region of interest is shown. *Scale bar*: 10 μm. (*B*) Stereological quantification of SIRT1-positive alveolar epithelial cells by the newCAST system. *Red*: positive stain; *black*: negative stain. (*C*) Representative blot for SIRT1 expression at the protein level in lung homogenate and its densitometric analysis. β-actin was used as loading control. (*D*) Representative lung sections stained with anti-p16 Ab (1:50). *Scale bar*: 20 μm. (*E*) Representative Western blots and densitometric analysis for p16 (1:200) and p21 (1:200) protein levels in lung homogenate and semiquantitative manual scoring of p16-positive alveolar epithelial cells. (*F*) Representative lung sections stained with antibody against β-galactosidase (1:50). *Scale bar*: 20 μm. *Red arrow* indicates β-gal–positive alveolar epithelial cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test, WT versus CARM1 heterozygous mice). Data are presented as mean ± SD. The experiments were repeated twice ($n = 4$–7).

The finding was corroborated by a significant 1.5-fold reduction of SIRT1 mRNA compared with scrambled control as analyzed by qRT-PCR (Figure 5D). Concomitantly, we observed a significant up-regulation of p16 and p21 but not p53 mRNA in siCARM1-transfected LA-4 cells (Figures 5E–5G). Furthermore, siCARM1-transfected cells showed a significantly higher percentage of β-galactosidase–positive cells compared with the scrambled control (50.57 ± 7.364% versus 2.210 ± 0.3404%; $P < 0.001$) (Figures 5H and 5I). The number of positive cells was comparable to a positive control where cells were treated with 100 μM H_2O_2 (data not shown). Because CS is the most common cause of emphysema, we further used CSE-treated, siCARM1-transfected LA-4 cells. In siCARM1-transfected cells stimulated with 5% CSE, the mRNA expression level of p21 was significantly up-regulated compared with unstimulated siCARM1-transfected cells ($P < 0.05$) (Figure 5J). In accordance with these data, we also observed elevated β-galactosidase activity in siCARM1-transfected LA-4 cells after CSE stimulation (Figure 5K), demonstrating that senescence in CARM1-deficient cells was further augmented after CSE stimulation. These results confirmed that CARM1 reduction triggered an accelerated senescence in ATII cells by attenuating the effect of SIRT1.

CARM1 Down-Regulation Leads to Impaired Wound Healing and Aberrant Differentiation

Given that the reduction of CARM1-induced senescence in ATII cells, we sought to determine the impact of CARM1 on alveolar epithelial cell function and repair capacity. We designed a wound-healing assay using siCARM1-transfected LA-4 cells where the cell monolayer was scratched to induce a wound. CARM1-deficient cells exhibited a significant decrease in proliferation and migratory distance as evidenced by a 72.7 ± 12.2% ($P < 0.001$) reduction in gap closure compared with scrambled siRNA (32.18 ± 0.9512% versus 8.769 ± 1.967%) (Figures 6A and 6B). This finding suggested that reduced CARM1 led to impaired wound healing of ATII-like cells.

In vivo repair mechanisms consist of cellular regeneration, migration, and differentiation. CARM1 was previously reported to regulate the differentiation of ATII into ATI cells in the embryonic E18.5 mouse lung (21). We explored the role of CARM1 in the differentiation of adult murine cells by culturing primary ATII cells from WT animals for 5 days under appropriate conditions. From Day 3, cells started to differentiate into ATI-like cells, as demonstrated by expression of the ATI cell marker expression T1α. During ATII to ATI cell transdifferentiation, CARM1 was significantly up-regulated as analyzed by qRT-PCR (Figure 6C). Similarly, we also isolated and cultured primary ATII cells from CARM1 heterozygous mice. We detected a significant down-regulation of T1α at the protein level at Day 5 in these mice compared with WT mice, indicating that CARM1 deficiency might lead to impaired differentiation (Figure 6D). This indicates that CARM1 expression is associated with alveolar epithelial cell transdifferentiation processes.

Finally, we analyzed the number of SP-C–positive cells in emphysematous mouse lungs. We observed an increased ratio of SP-C–positive cells to the total cell nuclei in elastase-treated CARM1 heterozygous mice compared with PBS-treated heterozygous mice (Figures 6E and 6F). Taken together, these results suggest that the higher number of ATII cells probably resulted from impaired transdifferentiation.

Discussion

The development of emphysema is purported to be influenced by premature aging/senescence of lung cells, but the underlying mechanism is unclear. Our study aimed at investigating the role of CARM1, a PRMT family member regulating cellular senescence via the antiaging protein/histone deacetylase SIRT1, in elastase-induced emphysema in WT and CARM1 heterozygous mice. We reported for the first time that CARM1 deficiency caused enhanced emphysema by accelerating senescence via SIRT1, leading to impaired regeneration and differentiation of alveolar epithelial cells.

To mimic the irreversible structural changes occurring in the lungs of patients with COPD even after smoking cessation (32), the elastase-induced emphysema mouse model is a useful and well-established tool. Despite its known half-life of a few hours and a turnover rate of 99% in 4 days (33, 34), elastase triggers a continuous airspace enlargement and lung function decline even after stimulus cessation (27, 35). Most recently, a study monitored and described emphysema progression for 12 weeks by using morphometry and microcomputed X-ray tomography (36). We used the elastase-induced emphysema model as an alternative to the CS model, which takes considerably more time to induce significant pathological changes in the lung (starting from 4 mo of exposure) (37). Furthermore, CS exposure of animals for up to 6 months only produces a mild disease, probably equivalent to human Global Initiative on Chronic Obstructive Lung Disease (GOLD) stage 1 or 2. In contrast, the elastase model is one of the fastest and easiest models available to study emphysema. This model can be established by a single treatment inducing progressive emphysema development that can reliably be monitored by lung function tests (35). Although the elastase model and the CS model have demonstrated pathologically relevant emphysematous changes, the differences in injury pathway are still not completely known. Furthermore, the onset and duration of lesions differ in both models. To complement our elastase model, we performed an *in vitro* study with CSE on siCARM1-treated LA-4 cells to evaluate the effect of CSE on cells with reduced CARM1. In addition, for a future study, we plan to use the CS model on SP-C–cre CARM1 mice.

In our study, we monitored elastase-induced emphysema progression in WT mice for a period of 161 days and demonstrate progressive disease severity associated with increased airspace enlargement and a decline in lung function parameters. Because CARM1 was shown to be indispensable for normal lung development, specifically for alveolar proliferation and differentiation (20, 21), we were interested in its role in emphysema progression. Interestingly, CARM1 was down-regulated in ATII cells of emphysematous lungs. CARM1 can be phosphorylated at a conserved serine residue (Ser-228 in human and Ser-229 in mouse), and the phosphorylation prevents CARM1 binding with the methyl donor S-adenosyl methionine, thus inhibiting its

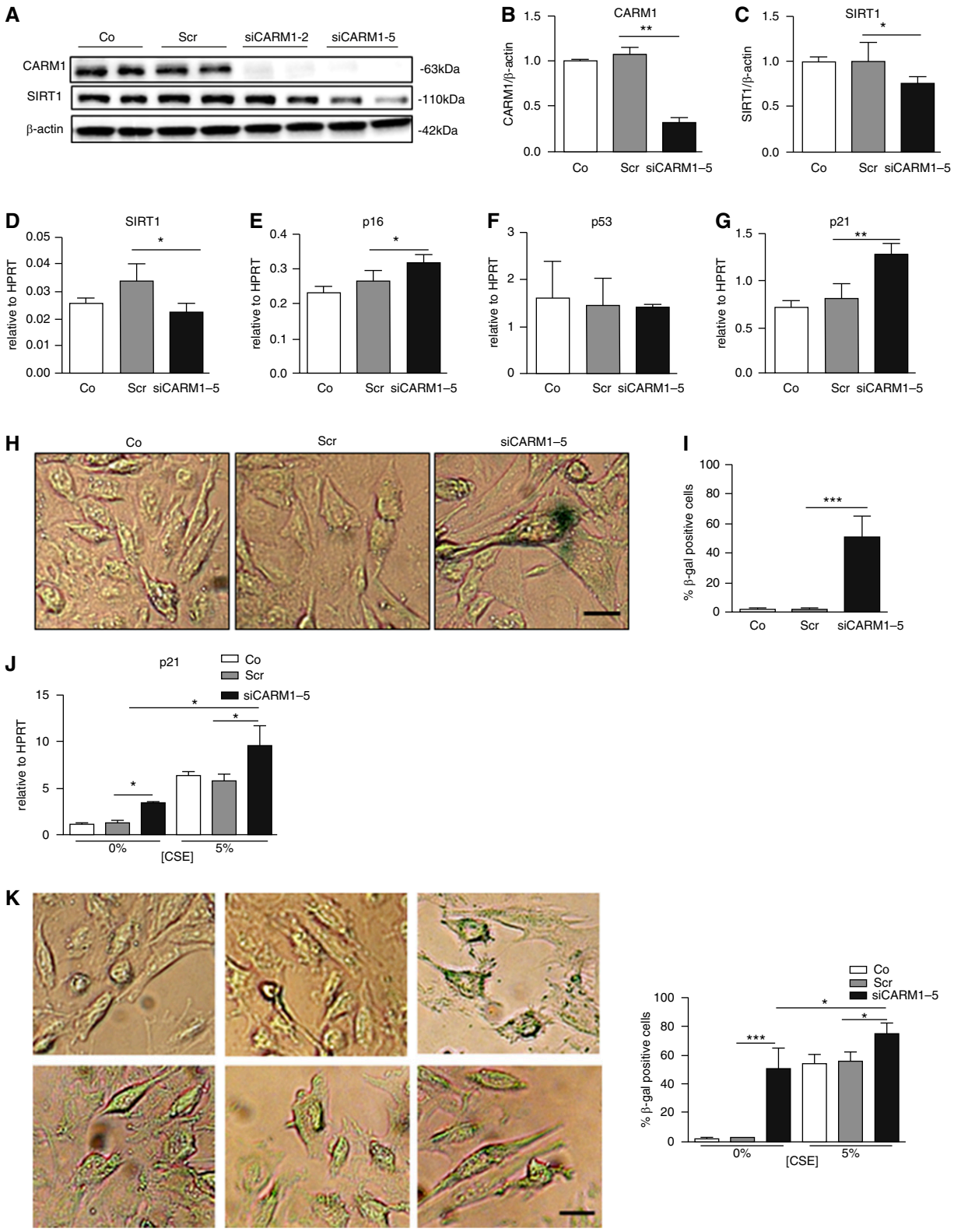

Figure 5. CARM1 reduction accelerated senescence in the ATII-like cell line LA-4. Cells were transfected with CARM1-specific small interfering (si)RNA (siCARM1–2 or siCARM1–5) or nonspecific scrambled siRNA (Scr) for 48 hours and harvested for RNA/protein analysis or incubated overnight with β-galactosidase staining solution. Untreated cells were taken as medium control (Co). (*A*) Representative Western blot showing CARM1 (*upper lane*) and SIRT1 (*middle lane*) at the protein level. β-Actin was used as loading control (*lower lane*). (*B* and *C*) Densitometric analysis of the blot for CARM1 (*B*) and

enzymatic activity (29, 38). We analyzed the ratio of phospho-CARM1 to CARM1 levels and observed a late effect on CARM1 phosphorylation after elastase treatment.

Furthermore, CARM1-deficient mice displayed pronounced and accelerated emphysema progression at Day 28 in response to elastase treatment. Increasing evidence suggests that aging is a significant risk factor for emphysema development (39). Aging/senescence is a stress response marked by a progressive decline in the function of multiple cells and tissues (40). Recently, the lungs of smokers with emphysema were associated with overexpression of the cellular senescence marker p16 and telomere shortening in both alveolar epithelial type II (ATII) and endothelial cells (41). Furthermore, deficiency in the antisenescence protein (SMP30) in mice promotes airspace enlargement after CS exposure (42). Most importantly, CARM1 is reported to be down-regulated in testis, thymus, and heart of 24-month-old aging rats (43). CARM1 reduction is also observed in replicative and H_2O_2-induced premature senescence of human diploid fibroblasts (44). Based on these reports and on our initial findings, we hypothesized that diminished CARM1 levels might promote enhanced emphysema by regulating cellular senescence. Therefore, we investigated SIRT1, an NAD+-dependent lysine deacetylase functioning in multiple cellular events but most importantly in the control of life-span and thus acting as an antisenescence gene (45). In the lungs of smokers or patients with COPD, SIRT1 is reported to be down-regulated (26, 46). In mice, SIRT1 deficiency led to enhanced emphysema in both elastase- and CS-induced models (25). Indeed, we observed a reduced basal level of SIRT1-positive alveolar epithelial cells in CARM1-deficient mice. CARM1 is reported to stabilize SIRT1 transcripts by CARM1-dependent methylation of HuR (23). We speculate that this intrinsic prosenescent status of alveolar epithelial cells observed in our study resulted from decreased stabilization of SIRT1. Our *in vitro* study showing SIRT1 down-regulation after siRNA-mediated CARM1 knockdown in LA-4 cells further confirmed the existence of a CARM1-SIRT1 axis in the regulation of senescence, particularly in ATII cells. These results were corroborated by an increased basal level of p16-positive alveolar epithelial cells (Figure 4) in CARM1-deficient mice and significant up-regulation of p16 as well as p21 in CARM1-deficient LA-4 cells (Figure 5). These are two of the widely used senescence markers known to increase with aging in several rodent and human tissues and most importantly in alveolar epithelial cells of patients with COPD (41, 47, 48). Moreover, previous studies showed CARM1-dependent post-translational methylation of HuR enhancing its association with p16 or p21 mRNA and leading to transcript degradation, whereas the absence of CARM1 led to transcript stabilization (22, 24, 49). The impact of CARM1-mediated regulation on senescence was confirmed by increased numbers of β-galactosidase–positive alveolar epithelial cells, which was augmented after CSE stimulation demonstrating that CARM1 deficiency could further enhance the senescence induction after injury stimulus in ATII cells.

CARM1-deficient mice were previously shown to have hyperproliferative immature ATII cells that could not differentiate into ATI (21). In contrast, in CARM1 knockdown LA-4 cells, we observed a reduced gap closure, suggesting that CARM1 was required for wound healing after an injury induction in the lung. Furthermore, at Day 5 when the isolated ATII cells were supposed to be completely differentiated into ATI cells under culture conditions, we detected a reduction of T1α at the protein level in CARM1-deficient mice compared with WT mice. Thus, we speculated that CARM1 deficiency could indeed lead to impaired transdifferentiation.

However, we also showed an increased number of SP-C–positive ATII cells in CARM1-deficient mice after elastase treatment (Figure 6), which could be related to hyperproliferation or dysregulated transdifferentiation of ATII into ATI, thereby leading to an accumulation of ATII cells. It is possible that CARM1 regulates transdifferentiation of ATII cells via the Wnt signaling pathway involved in alveolar epithelial transdifferentiation (50). Activation of Wnt/β-catenin signaling during lung injury promotes alveolar epithelial differentiation toward an ATI-like phenotype (51), and, recently, down-regulation of Wnt/β-catenin signaling has been implicated in parenchymal tissue destruction and impaired repair capacity in lungs of patients with COPD (52). The methyltransferase domain of CARM1 can specifically interact with β-catenin and thus acts as a *bona fide* coactivator for Wnt/β-catenin signaling (19), indicating that the CARM1-regulated Wnt pathway might be involved in alveolar epithelial differentiation. However, further study is required to elaborate how CARM1-regulated senescence could lead to impairment of alveolar epithelial transdifferentiation during emphysema development. Especially the upstream pathways regulating CARM1 via post-translational modifications might be a promising target for future investigation.

Taken together, our findings support the senescence hypothesis of emphysema development in COPD. In our current study, cellular senescence indeed contributed to the pathogenesis of emphysema via a CARM1–SIRT1 axis in ATII cells. We identified CARM1 as the

Figure 5. (Continued). SIRT1 (*C*) compared with β-actin. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test, siCARM1–5 versus Scr). (*D*) SIRT1 expression at the mRNA level. $^{*}P < 0.05$ (one-way ANOVA). (*E–G*) mRNA expression of p16 (*E*), p21 (*F*), and p53 (*G*). $^{*}P < 0.05$ and $^{**}P < 0.01$ (one-way ANOVA). (*H*) Representative images from β-galactosidase assay. *Scale bar*: 2 μm. (*I*) Quantification of β-galactosidase–positive cells from a total of 300 cells in 10 random fields per well in a 24-well plate. $^{***}P < 0.001$ (one-way ANOVA). Data were from three independent experiments. (*J*) The mRNA expression level of p21 inLA-4 cells treated with or without 5% of cigarette smoke extract (CSE). The cells were treated with CSE for 6 hours. (*K*) Representative images of β-galactosidase assay and quantification of β-gal–positive cells treated with siCARM1 and/or 5% CSE. *Scale bar*: 2 μm. $^{*}P < 0.05$ and $^{***}P < 0.001$ (Student's *t* test versus siCARM1 treated with 5% CSE). Data are presented as mean ± SD. Data were from two independent experiments.

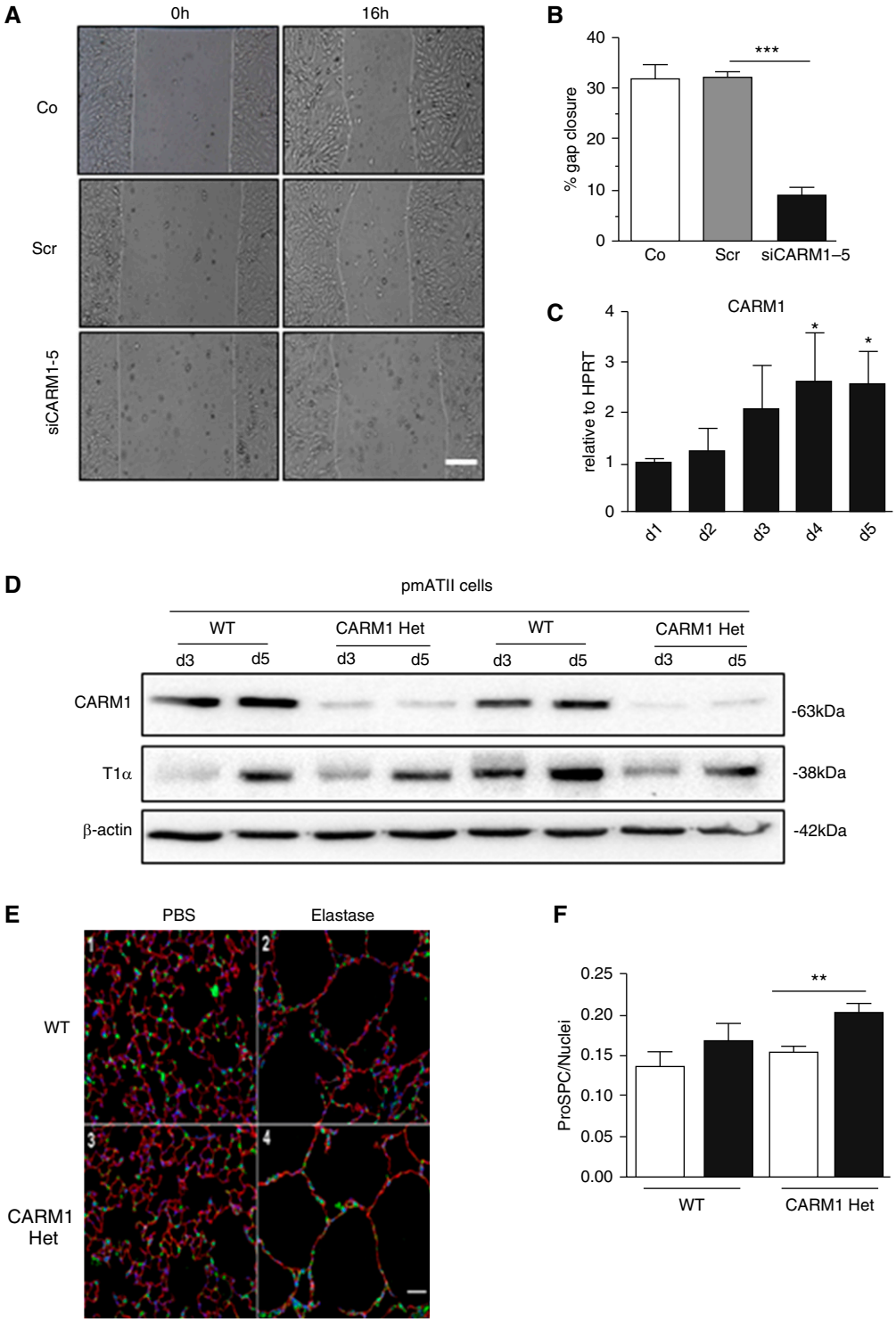

Figure 6. CARM1 reduction impaired wound healing in LA-4 cells and transdifferentiation of alveolar epithelial cells. (*A*) Wound healing assay performed in confluent cultures of siCARM1–5 transfected LA-4 cells using a pipette tip. *Scale bar*: 200 μm. (*B*) Quantification of gap closure determined 16 hours after injury. ***$P < 0.001$ (one-way ANOVA). Data were from three independent experiments. (*C*) CARM1 mRNA expression during differentiation of isolated primary ATII (pmATII) cells into an ATI-like phenotype in culture at the indicated days (d). *$P < 0.05$ (one-way ANOVA, Dunnett's multiple comparison test versus Day 1; $n = 3$). (*D*) Representative Western blot for protein levels of CARM1 and T1α at the indicated days of culture of isolated primary ATII cells from WT and CARM1 heterozygous animals. (*E*) Representative immunofluorescence images showing SP-C–positive (*green*) alveolar epithelial cells on lung sections from WT or CARM1 heterozygous mice treated with either PBS or elastase. DAPI (*blue*) used as nuclear staining. *Scale bar*: 50 μm. (*F*) Stereological quantification of SP-C–positive alveolar epithelial cells to total number of nuclei. **$P < 0.01$ (one-way ANOVA, PBS versus elastase-treated CARM1 heterozygous animals). Data are presented as mean ± SD. Data are from two independent experiments.

upstream regulator of SIRT1, thereby regulating senescence and affecting regeneration, repair, and differentiation of ATII cells. Our findings also provide an alternative strategy to treat emphysema. Because there is no current treatment to reverse the damaged lung structure seen in emphysema, arresting emphysema progression would be a more realistic goal. Pharmacological intervention might be applied to induce CARM1 or to prevent its degradation in emphysematous lungs. Thus, CARM1 may prove to be an effective target to slow the premature lung aging process observed in patients with COPD. ■

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