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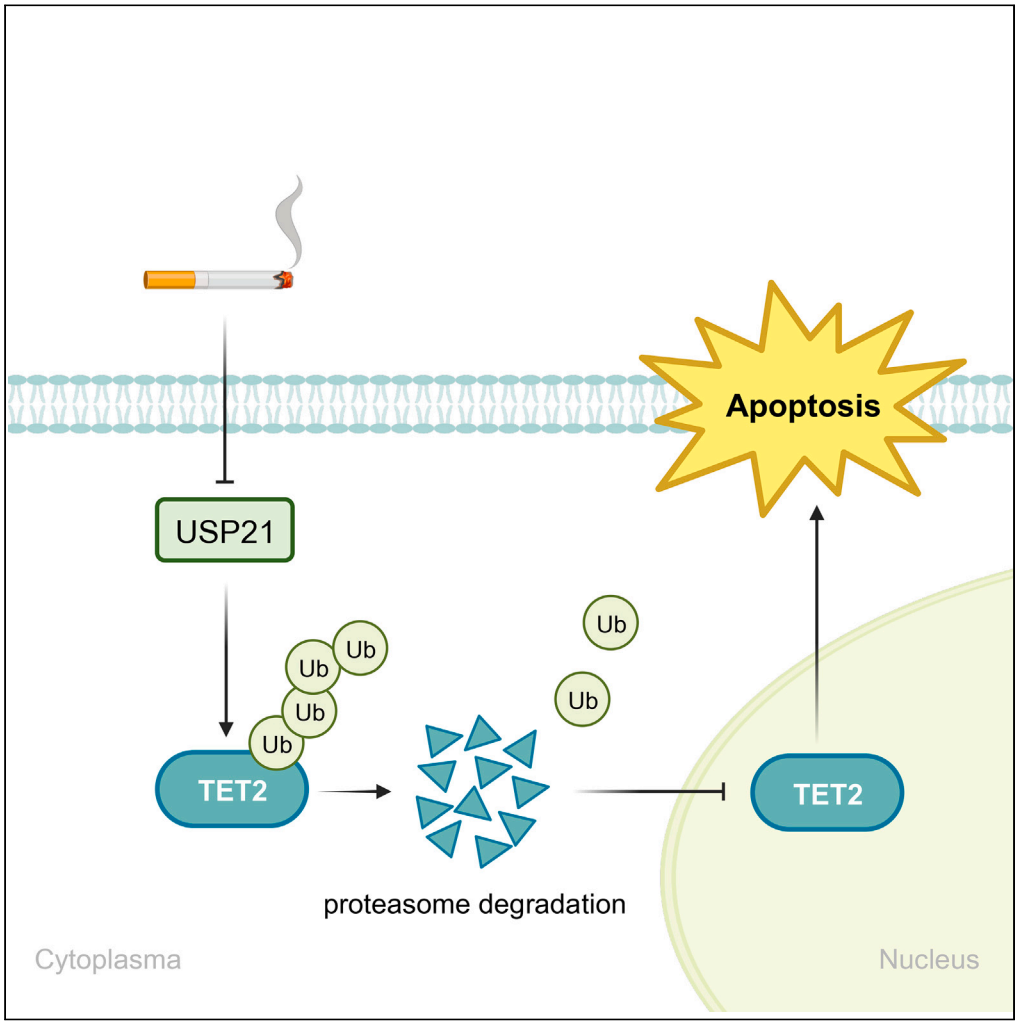

Lijuan Luo, Zihang Zeng, Tiao Li, ..., Yongguang Tao, Yi Li, Yan Chen

503191@csu.edu.cn (Y.L.)
chenyan99727@csu.edu.cn (Y.C.)

Highlights

TET2 is a reduction in protein levels but not RNA levels in COPD

TET2 inhibited CS-induced apoptosis of lung epithelial cell *in vivo* and *in vitro*

USP21 stabilizes TET2 expression in CS-induced lung epithelial cells

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TET2 stabilized by deubiquitinase USP21 ameliorates cigarette smoke-induced apoptosis in airway epithelial cells

Lijuan Luo,[1,2,3,4,9] Zihang Zeng,[1,2,3,4,9] Tiao Li,[8] Xiangming Liu,[1,2,3,4] Yanan Cui,[1,2,3,4] Yongguang Tao,[5,6] Yi Li,[7,*] and Yan Chen[1,2,3,4,10,*]

SUMMARY

DNA demethylase TET2 was related with lung function. However, the precise role of TET2 in cigarette smoke (CS)-induced apoptosis of airway epithelium cells, and the mechanisms involved, have yet to be elucidated. Here, we showed that CS decreased TET2 protein levels but had no significant effect on its mRNA levels in lung tissues of chronic obstructive pulmonary disease (COPD) patients and CS-induced COPD mice model and even in airway epithelial cell lines. TET2 could inhibit CS-induced apoptosis of airway epithelial cell *in vivo* and *in vitro*. Moreover, we identified ubiquitin-specific protease 21 (USP21) as a deubiquitinase of TET2 in airway epithelial cells. USP21 interacted with TET2 and inhibited CSE-induced TET2 degradation. USP21 downregulated decreased TET2 abundance and further reduced the anti-apoptosis effect of TET2. Thus, we draw a conclusion that the USP21/TET2 axis is involved in CS-induced apoptosis of airway epithelial cells.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation and reduced gas exchange due to abnormalities of the airways and alveoli.[1] COPD is among the top three causes of death worldwide, and it's estimated that 4.41 million people will die of COPD every year by 2040.[2] as such, COPD poses a huge burden on the economy and society. Therefore, elucidating the pathogenesis is the most important thing in the prevention and treatment of COPD.

The main risk factor for COPD is the inhalation of noxious particles, such as cigarette smoke (CS), air pollutants, and job-related exposures.[1] CS-induced apoptosis of airway epithelial cells was considered to lead to emphysema, which is the basic pathological change of COPD.[3–5] The cleaved caspase3 is an important indicator of apoptosis, while intratracheal injection of active caspase3 is sufficient to cause emphysema in mice.[6] What's more, we previously demonstrated that CS participates in emphysema partially through the downregulation of gene encoding apoptosis inhibitors (BCL2). The BCL2, The B cell lymphoma 2 (BCL2) family member, which serves an important role in apoptosis suppression. We have already found that epigenetic alternation played a role in this deregulation of BCL2 expression. Epigenetic changes have been shown to strongly correlate with the development of COPD.[7] Epigenetic changes the heritable state of gene expression and chromatin organization without modifying nucleotide sequences, included histone modifications, DNA methylation, and non-coding RNA.[8] Among them, DNA methylation has been reported that association with the progress of COPD.[7] A large-scale epigenome-wide meta-analyses indicated that DNA methylation is related with the pulmonary function.[9]

Tet methylcytosine dioxygenase 2 (TET2) is one of the key enzymes mediating demethylation of DNA, which involved in the physiological processes; include inflammation, autophagy, oxidative stress, immunity, and apoptosis.[10–14] There have been reported that TET2 deficiency can accelerate cell apoptosis in multiple cancer cell types.[15] TET2 can enhance intracellular connections to maintain airway epithelial cells integrity at the early stage of pneumonia.[16] Lacking TET2 in mouse blood cells exacerbated the development of emphysema and inflammation after treatment of CS exposure.[17] In addition, a COPD genetic association study in UK Biobank have shown that TET2 can participate in

[1]Department of Pulmonary and Critical Care Medicine, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China
[2]Research Unit of Respiratory Disease, Central South University, Changsha, Hunan 410011, China
[3]Diagnosis and Treatment center of Respiratory Disease, Central South University, Changsha, Hunan 410011, China
[4]Clinical Medical Research Center for Pulmonary and Critical Care Medicine in Hunan Province, Changsha, Hunan 410011, China
[5]Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Department of Pathology, Xiangya Hospital, Central South University, Changsha, Hunan 410078, China
[6]NHC Key Laboratory of Carcinogenesis (Central South University), Cancer Research Institute and School of Basic Medicine, Central South University, Changsha, Hunan 410078, China
[7]Department of Infectious Disease Department, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China
[8]Department of Pulmonary and Critical Care Medicine, Xiangya Hospital, Central South University, Changsha, Hunan 410011, China
[9]These authors contributed equally
[10]Lead contact
*Correspondence: 503191@csu.edu.cn (Y.L.), chenyan99727@csu.edu.cn (Y.C.)
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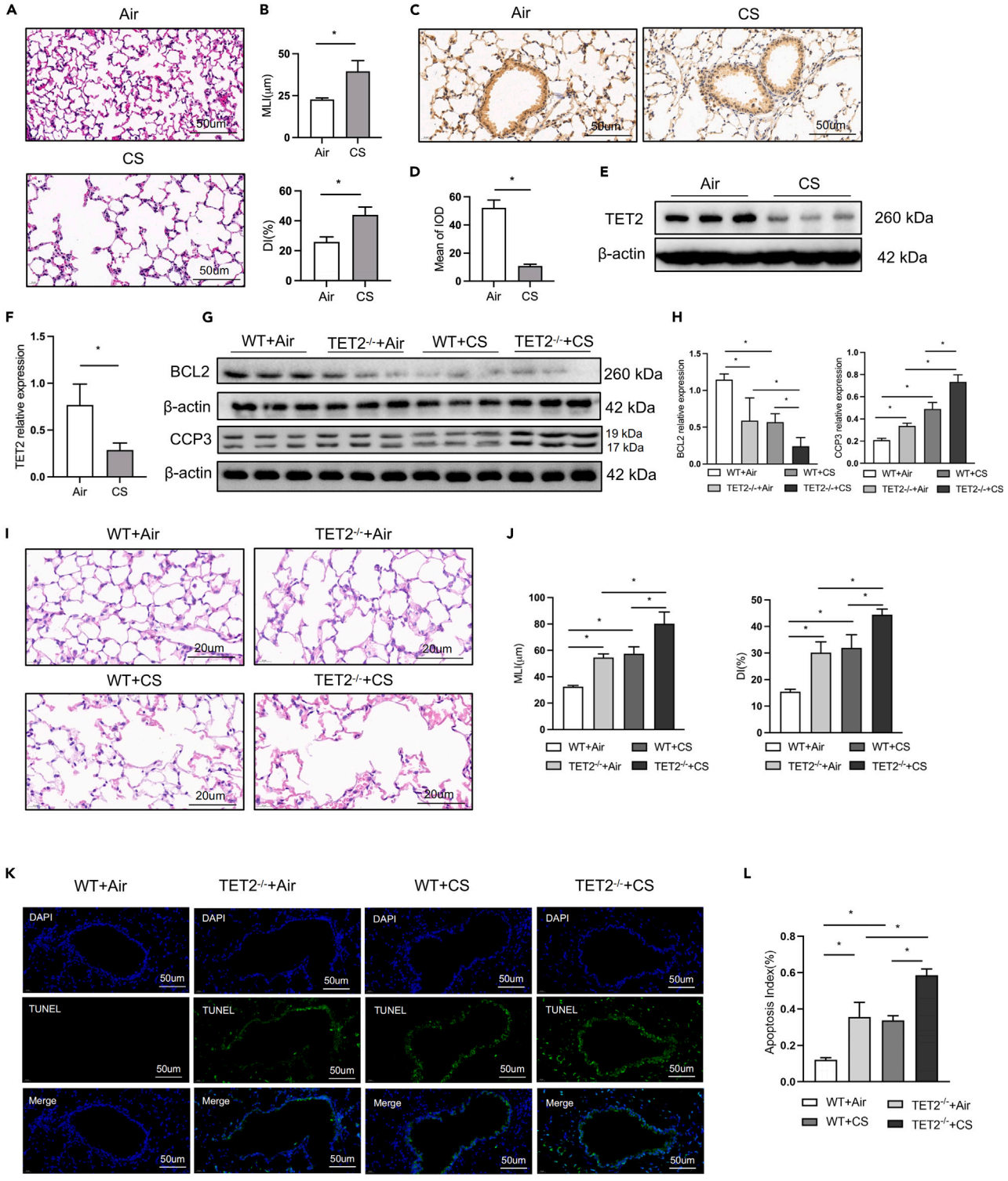


Figure 1. TET2 Knockout enhanced the apoptosis of CS-induced COPD mouse model

(A) HE staining of mice lung tissue (scale bar: 50μm; inserts show the expanded image of the indicated region); (B) Morphometric measurements of MLI (μm), Morphometric measurements of DI (%).

(C) IHC staining of TET2 in the mice lung tissue (scale bar: 50μm; inserts show the expanded image of the indicated region); (D) The mean IOD of TET2 in the mice lung tissue; (E). Western blot of TET2 protein expression in the lung tissue of mice; (F) Densitometry analysis of TET2 protein expression; (G) Western blot of BCL2, cleaved caspase3 (CCP3) and β-actin protein expression in the lung tissue of mice; (H) Densitometry analysis of BCL2 and cleaved caspase3(CCP3) protein

Figure 1. *Continued*

expression; (I) HE staining of mice lung tissue with different treatment (scale bar: 20μm; inserts show the expanded image of the indicated region); (J) Morphometric measurements of MLI (μm), Morphometric measurements of DI (%); (K) TUNEL assay of lung tissue (scale bar: 50μm; inserts show the expanded image of the indicated region); (L) Apoptosis index of lung tissue by TUNEL assay. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, compared between the marked groups.

the fetal lung development and associate with lung function,[18] suggesting that TET2 may be a risk factor for COPD. However, the function of TET2 in COPD airway epithelial cell remains unclear, and little information is focused on the regulatory mechanisms of TET2 protein level.

The ubiquitin proteasome system (UPS) is one of the principal protein degradation pathways in eukaryote. Protein degradation by the UPS proceeds from the ligation of one or more ubiquitin molecules to a specific target protein catalyzed by E1, E2, and E3 enzymes and then target protein will be recognized and cleaved by the 26S proteasome.[19] Ubiquitination is regarded as a signal to degradation targets. Under normal conditions, ubiquitination is a reversible process regulated by deubiquitinase (DUBs). DUBs remove ubiquitin from substrate proteins and protect proteins from proteasomal degradation to maintain the balance of UPS.[20] There have researches indicated that the increase of apoptosis in CS-induced emphysema may be the result of destruction of the UPS.[21] What's more, cancerous cells are inhibited from proliferating and induced to apoptosis by targeting UPS.[22,23] In addition, there have some researches pay attention to the role of post-translational modifications in modulating TET2 activity recently, including ubiquitination,[24] phosphorylation,[25] and acetylation.[26] It has been shown that regulating the protein stability of TET2 can protects against obesity-induced impairment of the skeletal muscle function.[27] Here, we considered that TET2 protein might be regulated by ubiquitin proteasome pathway and then performed a DUB screen for the regulator of TET2. We identified ubiquitin-specific peptidase 21 (USP21) as a potent DUB for TET2 deubiquitination and postulated that CS promoted airway epithelial apoptosis by the inhibition of USP21/TET2 axis. These results provide great evidence for the regulation of TET2 protein stability by CS in the pathogenesis of COPD.

RESULTS

TET2 knockout enhanced the apoptosis of CS-induced COPD mouse model

Firstly, we establish an CS exposed COPD animal model, HE staining of lung tissue were showed that the alveolar airspace enlargement and the alveolar wall was destroyed, which were more obvious in CS exposure group than control group (Figure 1A). In addition, we found pathomorphological indicators the mean linear intercept (MLI) and destructive index (DI) in CS group were significantly higher than Air group (Figure 1B). Next, we test TET2 by IHC and western blot (WB). From the results, TET2 was decreased in the lung tissue of CS-induced COPD model, and it was mainly located in airway epithelial cells (Figures 1C and 1E). What's more, quantitative analysis showed that there was statistical significance between the two groups (Figures 1D and 1F). Furthermore, to understand the role of downregulated TET2 in COPD, we used TET2 gene knockout mice ($TET2^{-/-}$) to construct an animal model of CS exposed COPD. As shown in Figures 1I and 1J, TET2 knockout mice exhibited alveolar enlargement and alveolar destruction regardless of the presence or absence of CS. However, the severity of this phenomenon was further exacerbated in the presence of smoking. Furthermore, we observed that TET2 knockout can reduce the expression of anti-apoptotic protein BCL2 and increase the level of pro-apoptotic protein cleaved caspase3, suggesting that reduced TET2 can promote CS-induced apoptosis (Figures 1G and 1H). While we also found the same alteration in TUNEL assay that TET2 knockout can significantly augment the apoptosis of lung tissue, especially in airway epithelial cells, compared with the control group (Figures 1K and 1L).

Reduction of TET2 promote the CSE-induced apoptosis in BEAS-2b cells

From previous results, we found that TET2 protein is mainly expressed in the airway epithelium. And then we use siRNA targeting TET2 to interfere with BEAS-2b cells.

As shown in Figure 2A, TET2 was significantly reduced in siTET2 group than scramble group, which indicated that knockdown of TET2 by siRNA was successfully. We found that lack of TET2 also increased the pro-apoptotic protein of cleaved caspase3 and severely decreased the anti-apoptotic protein of BCL2 in BEAS-2b cells (Figures 2A and 2B). In addition, we proved that knockdown of TET2 enhanced cell apoptosis in BEAS-2b through the flow cytometry assay (Figures 2C and 2D). Aforementioned results further indicated that TET2 silence promoted lung epithelial cell apoptosis. TET2 is an important DNA demethylase that regulates gene methylation. We hypothesized that TET2 regulates cell apoptosis through its demethylase activity. To clarify this, we applied methylation inhibitor 5-AZA to BEAS-2b cells and found that knockdown of TET2 can promote the expression of cleaved caspase3, while 5-AZA treatment can restore the expression of cleaved caspase3 (Figures 2E and 2F). This result indicated that TET2 regulates cigarette smoke extract (CSE) induce cell apoptosis may through modulating cleaved caspase3 demethylation.

Overexpression of TET2 attenuate the CSE-induced apoptosis in BEAS-2b cells

To verify whether TET2 could prevent the epithelia apoptosis, we overexpressed TET2 in BEAS-2b cells treated with CSE. As expected, CSE increased cleaved caspase3 and decreased BCL2 levels. TET2 overexpression rescued these effects, reducing cleaved caspase3 and increasing BCL2 levels after CSE treatment (Figures 3A and 3B). What's more, flow cytometry assay demonstrates that the apoptosis of BEAS-2b was obviously elevated under CSE stimulation. However, overexpression of TET2 alleviated the epithelia apoptosis after CSE exposure (Figures 3C and 3D). These results reflected that TET2 can prevent CSE-induced apoptosis in epithelial cells.

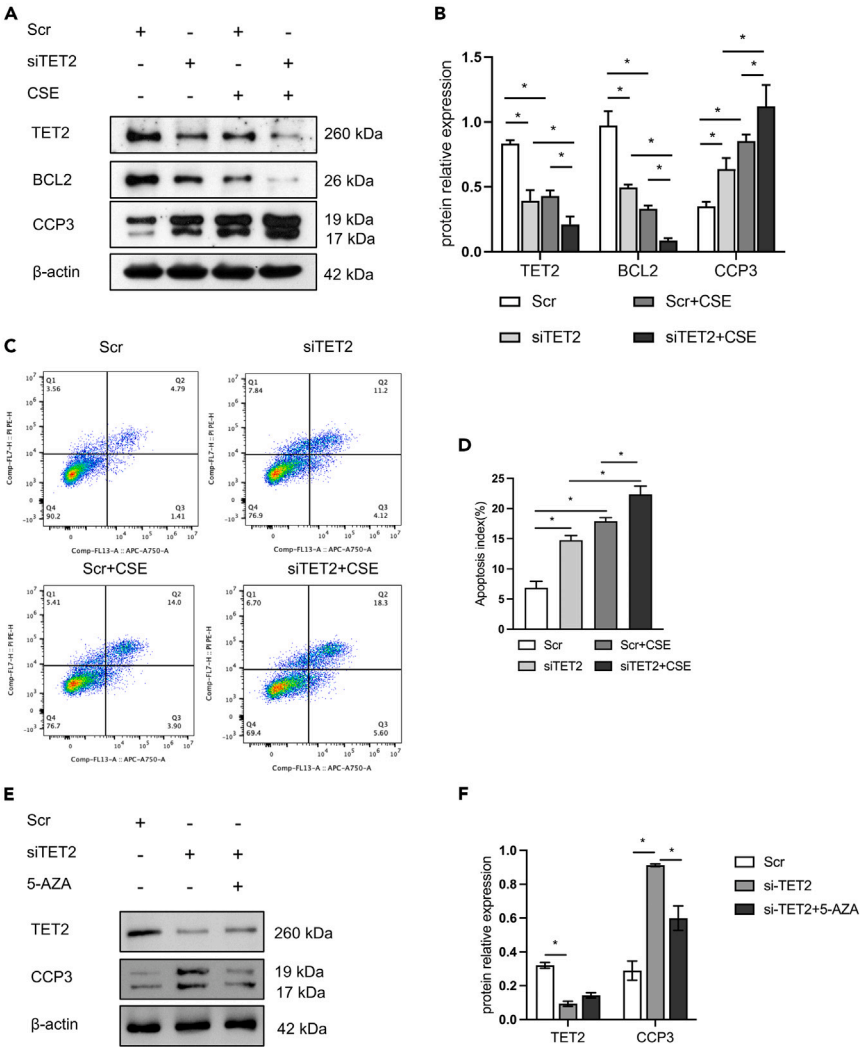


Figure 2. Reduction of TET2 promote the CSE induced apoptosis in BEAS-2b cells

(A) Western blot of TET2, BCL2, cleaved caspase3(CCP3) and β-actin protein expression in the BEAS-2b cells; (B) Densitometry analysis of TET2, BCL2 and cleaved caspase3(CCP3) protein expression; (C) Flow cytometry for detecting cell apoptosis; (D) Apoptosis index between different groups; (E) Western blot of TET2, cleaved caspase3(CCP3) and β-actin protein expression in the BEAS-2b cells. Scr: BEAS-2b cells exposed to non-targeting negative control siRNA. siTET2: BEAS-2b cells exposed to siTET2; (F) Densitometry analysis of TET2 and cleaved caspase3(CCP3) protein expression. Scr: BEAS-2b cells exposed to non-targeting negative control siRNA. siTET2: BEAS-2b cells exposed to siTET2; CSE: BEAS-2b cells exposed to CSE (5% CSE at 24 h). 5-AZA: BEAS-2b cells treated with 5-AZA (5uM). Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, compared between the marked groups.

CSE inhibit TET2 protein expression via the ubiquitin proteasome pathway

RNA expression data were downloaded from the GEO datasets, TET2 mRNA expression levels in bronchial brushings were similar between nonsmokers, smoker, and COPD (Figure 4A). We found that the expression of TET2 was decreased in lung tissue of COPD patients, but there was no difference in mRNA level between groups (Figures 4B–4D). CS exposure showed no significant effect on TET2 mRNA level in mouse lung tissues (Figure 4E). These results suggesting that the decrease in TET2 protein in COPD was not mediated at the transcription stage. Therefore, we speculate that whether TET2 is regulated by the ubiquitin proteasome pathway which is a route of protein degradation in eukaryotes. Next, we use proteasome inhibitors MG132 and protein synthesis inhibitor cycloheximide (CHX), respectively, into BEAS-2b cells. The results showed that TET2 was diminished in a time-dependent manner with CHX treatment, and we further found that proteasome inhibitor MG132 stabilized the expression of TET2 (Figures 4F and 4G). These data suggested that TET2 may be degraded via proteasome mechanism.

USP21 deubiquitinates TET2 and inhibits its proteasomal degradation

To further explore the mechanism of TET2 degradation by ubiquitin proteasome, previous research indicated the important role of USP49 in COPD.[28] Then, we found a suitable dataset, GSE3320, in the GEO database, which covers the expression profiles in the small airway epithelial

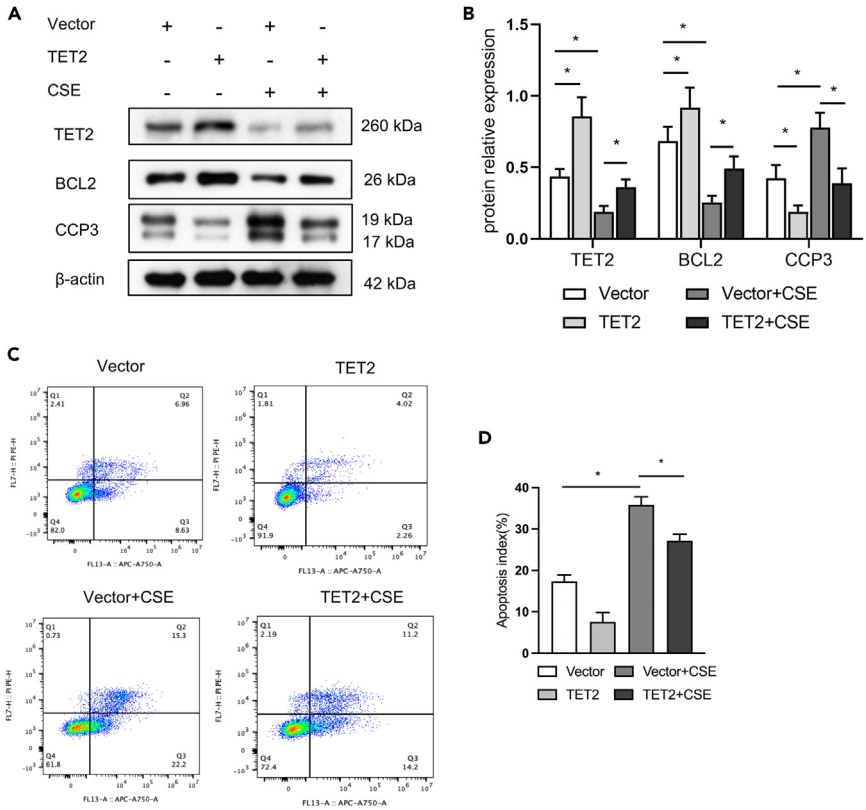


Figure 3. Overexpression of TET2 attenuate the CSE induced apoptosis in BEAS-2b cells

(A) Western blot of TET2, BCL2, cleaved caspase3(CCP3) and β-actin protein expression in the BEAS-2b cells. Vector: BEAS-2b cells exposed to negative control plasmid. TET2: BEAS-2b cells exposed to overexpression plasmid of TET2; CSE: BEAS-2b cells exposed to CSE (5% CSE at 24 h); (B) Densitometry analysis of TET2, BCL2 and cleaved caspase3(CCP3) protein expression; (C) Flow cytometry for detecting cell apoptosis; (D) Apoptosis index between different groups. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, compared between the marked groups.

cells of smokers. We individually examined the expression of each molecule in the USP family and found statistically significant differences only for USP21, 22, 25, and 46 (Figure S1). Finally, we used a heatmap to systematically present the expression profiles of USPs because it provides a comprehensive visual representation (Figure 5A). To determine whether the plasmid was successfully transfected, we added FLAG tags to display the degree of plasmid transfection. Then we transfected these plasmids of the previous molecules in BEAS-2b cells to observe the influence of these on TET2 expression. Among them, USP21 remarkably upregulated the TET2 level with a lower concentration than other plasmids (Figure 5B). In turn, we gradient transfected USP21 plasmid in BEAS-2b cells, TET2 was increased with the elevated of transfected plasmid concentration (Figure 5C). In addition, CSE also reduce the level of USP21 protein in epithelial cells (Figure 5D). Moreover, immunofluorescent staining and co-immunoprecipitation proved that USP21 can interact with TET2 (Figures 5E and 5F). Next, to prove that USP21 could promote TET2 stability, CHX or MG132 was applied to the BEAS-2b cells after modulating of USP21 to detect the stability of TET2. The results demonstrated that TET2 protein level decreased more slowly in USP21 overexpressed cells than in the control cells with CHX treatment (Figure 5G). In addition, we added MG132 to control cells and cells depleted of USP21. The TET2 protein level increased more slowly after silencing USP21 (Figure 5H). Furthermore, ubiquitination assay showed that the ubiquitination level of TET2 in the shUSP21 group was much higher than that in the control group (Figure 5I). Collectively, our data indicated that USP21 specifically stabilized TET2 protein in BEAS-2b cells.

Inhibition of TET2 protein degradation protects CSE-induced apoptosis in BEAS-2b cells

To understand if CSE induced TET2 reduction is mediated by proteasome, we applied CSE and MG132 to cells, which demonstrated that MG132 treatment rescued the TET2 expression in CSE treated cell and increased the expression of anti-apoptotic protein BCL2, reduced the expression of pro-apoptotic protein cleaved caspase3 and decreased the cell apoptosis after CSE treatment (Figures 6A–6D). Silencing USP21 reduced TET2 levels, increased cleaved caspase3 expression, decreased BCL2 expression, and promoted apoptosis in CSE treated cells. However, MG132 intervention increased TET2 and BCL2 expression, decreased cleaved caspase3 expression, and inhibited apoptosis in USP21 silenced cells exposed to CSE (Figures 6E–6H). In addition, to further test whether USP21 ameliorates apoptosis through regulating TET2 expression in CSE exposed cells, USP21 shRNA and TET2 overexpressed plasmid were simultaneously applied to treat bronchial epithelial cells. The decreased levels of BCL2, increased level of cleaved caspase3 and enhanced cell apoptosis induced by the depletion of USP21

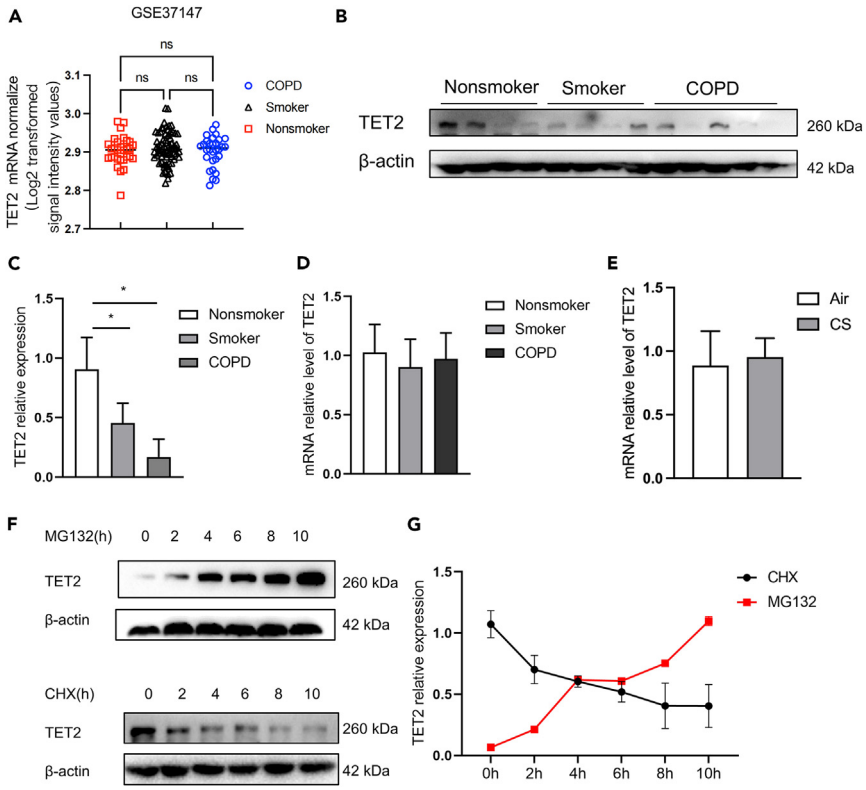


Figure 4. CSE inhibit TET2 protein expression via the ubiquitin proteasome pathway

(A) TET2 mRNA levels in bronchial brushings obtained from GEO Profiles (GSE37147, https://www.ncbi.nlm.nih.gov/geoprofiles); (B) Western blot of TET2 in lung tissue of COPD patients; (C) Densitometry analysis of TET2 protein expression; (D) qPCR of TET2 mRNA expression in the lung tissue of COPD patients; (E) qPCR of TET2 mRNA expression in the mice lung tissue; (F and G) CHX or MG132 was subjected to BEAS-2b cells. Data are presented as the mean ± SD of three independent experiments. *p < 0.05, compared between the marked groups.

could be rescued by the overexpression of TET2 in CSE treated cells (Figures 6I–6L). These findings demonstrated that USP21/TET2 axis played an important role in regulating CSE-induced apoptosis of airway epithelial cells.

DISCUSSION

In the current study, we demonstrated that TET2 expression was reduced following CS treatment in airway epithelia cells, and the lung tissue of both mice and patients. Functionally, we found that TET2-mediated cell apoptosis to drive the pathogenesis of COPD when induced by CS. Mechanistically, we found that deubiquitinating enzyme USP21 interacted with TET2 and inhibit TET2 ubiquitination and degradation in the epithelia cells and then alleviate CSE-induced apoptosis. Collectively, these data demonstrated that TET2 could be considered as a new interventional target for COPD.

Methylation played a key effect in COPD and various methylases have been reported to participate in the regulation of COPD. Coactivator-associated arginine methyltransferase-1 (CARM1) regulates airway epithelial cell injury in COPD progression.[29] Complete loss of CARM1 leads to disrupted differentiation and maturation of epithelial cells and promote the development of emphysema.[30] Our previous work also shown that dysregulation of DNA methyltransferase DNMT1 involved in the pathogenesis of COPD.[31,32] However, there is less research to explore the function of DNA demethylase in COPD. Surprisingly, a genetic association study in UK Biobank based on a large sample reported that the mutation of DNA demethylase TET2, as a newly discovered suspect site, may be contributed to the pathogenesis of COPD.[18] In present study, we found that the expression of TET2 was obviously downregulated in the lung tissue of CS exposed COPD animal model and COPD patients. In addition, TET2 was expression mainly in airway epithelial cells from the result of IHC. Combined with previous studies, these data also proved that TET2 was dysregulated in COPD.

Enhanced apoptosis is widely accepted as one of the most important mechanisms in COPD development.[4] Anti-apoptotic protein BCL2 was downregulated in COPD patients.[31] Previous study has shown that TET2 deficiency facilitates tumor cell apoptosis.[15] Another research demonstrated that mice carring TET2 knockout hematopoietic cells showed significantly increased development of emphysema with CS treatment.[17] Contrary to previous research, we hypothesized that TET2 may contribute to CS-induced emphysema by promoting apoptosis in bronchial epithelial cells. Consistent with this hypothesis, we found that TET2 is predominantly localized in bronchial epithelial cells and TET2 knockout enhanced apoptosis in lung tissue through reduced anti-apoptotic protein BCL2 and increased pro-apoptotic protein cleaved caspase3.

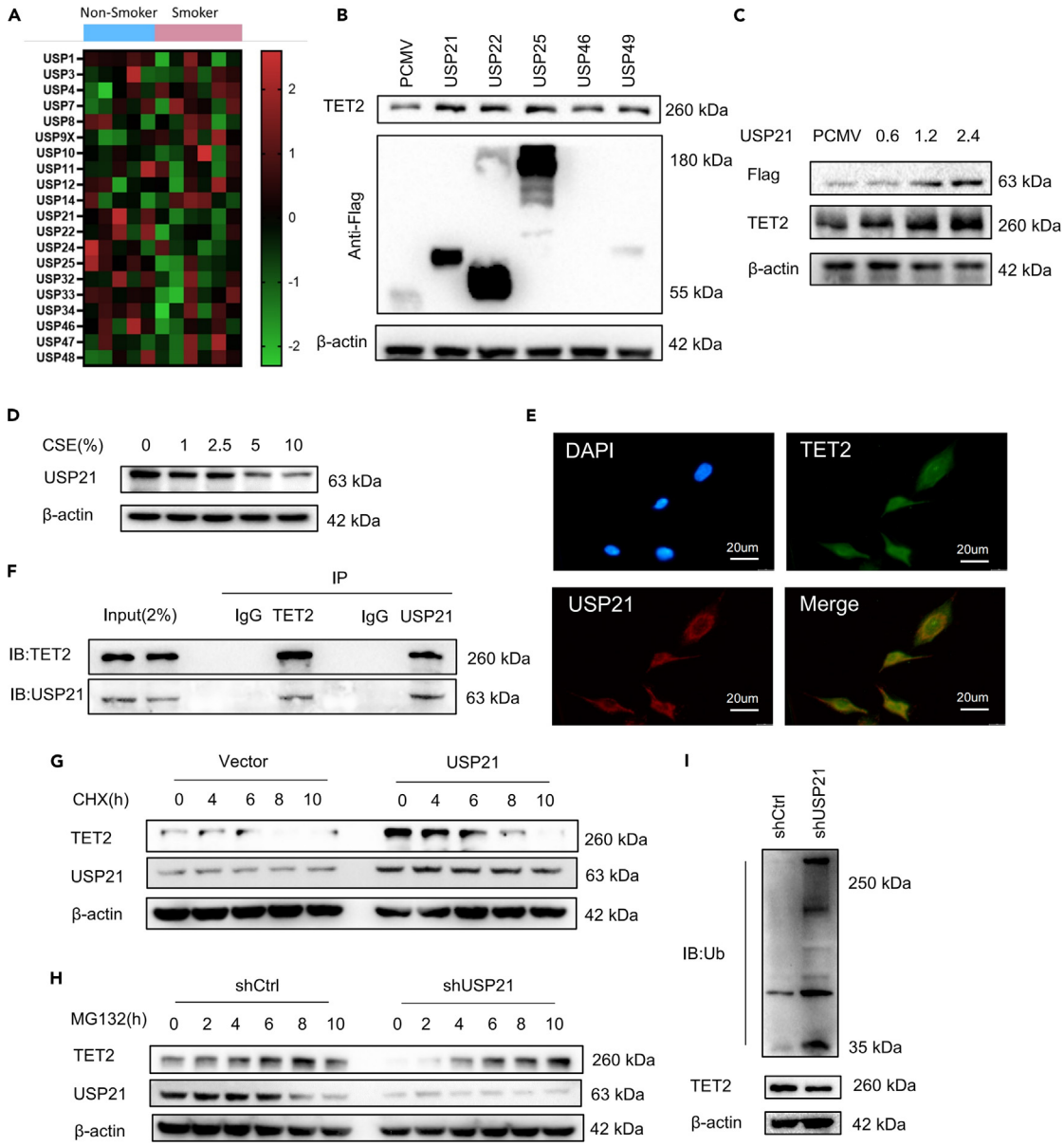
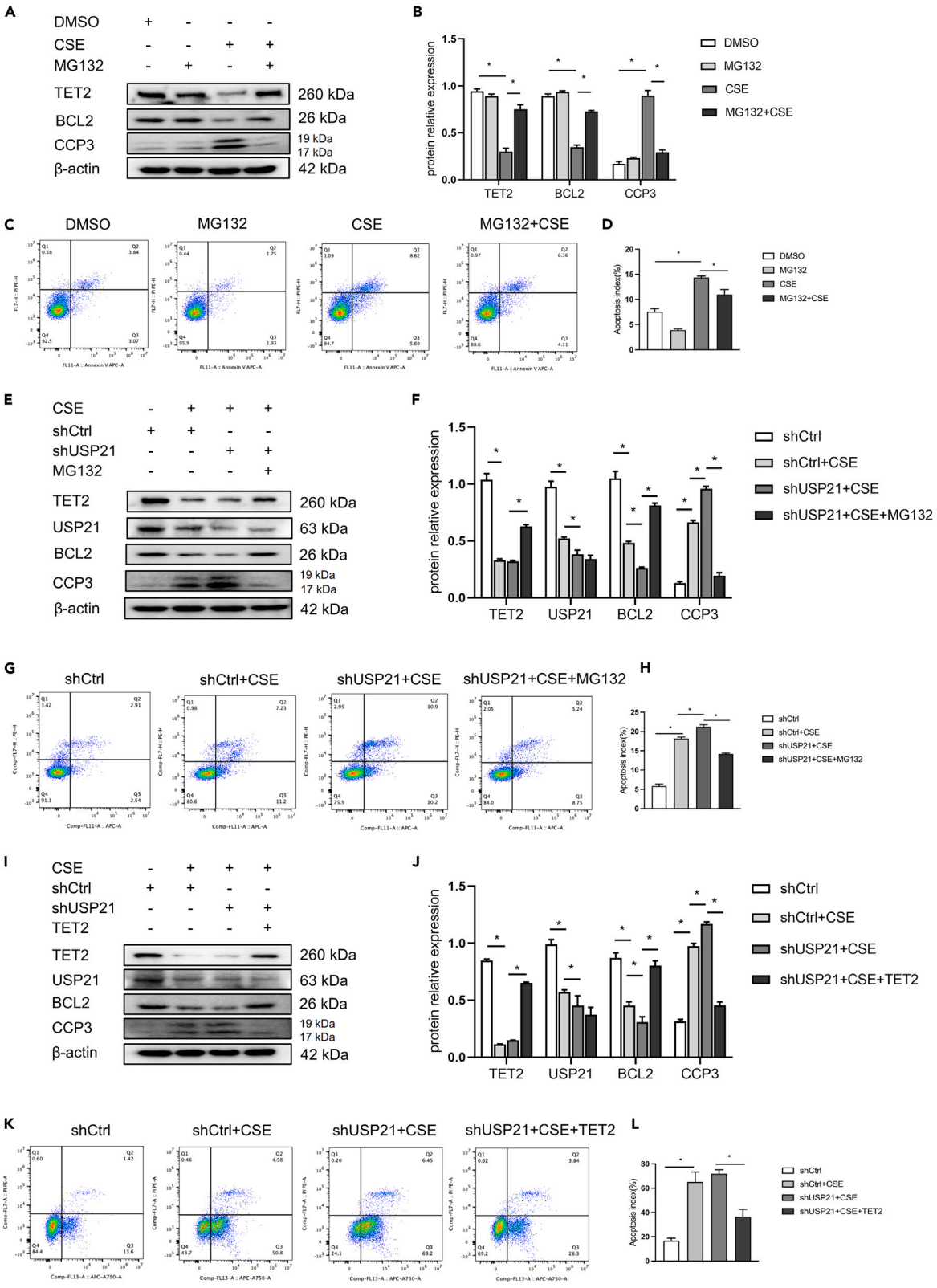


Figure 5. USP21 deubiquitinates TET2 and inhibits its proteasomal degradation

(A) Bioinformatics analysis from GEO database (https://www.ncbi.nlm.nih.gov/geoprofiles); (B) Deubquitinase screen for TET2; (C) Western blot of Flag,TET2 and β-actin protein expression in the BEAS-2b cells with USP21 plasmid treatment; (D) Western blot of USP21 and β-actin protein expression in the BEAS-2b cells with CSE treatment; (E) Immunofluorescence stanning of TET2 (green), USP21 (red) and DAPI (blue) and merged image(scale bar: 20μm; inserts show the expanded image of the indicated region); (F) Immunoprecipitation of TET2 and USP21, the ratio of immunoprecipitated TET2 and USP21 to the input is 2%; (G) CHX were applied to cells after USP21 overexpression; (H) MG132 were applied to cells after USP21 knockdown; (I) Ubiquitination assay of TET2 after USP21 knockdown. Data are presented as the mean ± SD of three independent experiments.

Additionally, TET2 knockout mice exhibited more severe emphysema, as indicated by higher MLI and DI, in the CS-induced COPD model. Furthermore, we demonstrated that knocking down TET2 enhanced CSE-induced apoptosis in epithelial cells. Collectively, our result suggests that reduced TET2 promotes CS-induced emphysema in COPD by increasing apoptosis of bronchial epithelial cells. Based on TET2 is an important DNA demethylase that regulates gene methylation through its enzymatic activity. It is possible that TET2, through its enzymatic activity, influences the methylation status of specific genes or regulatory regions involved in apoptotic signaling pathways. And in our study, we found that the expression of cleaved caspase3 was restored after silencing TET2 with methylation inhibitor 5-AZA intervention. A plausible explanation for TET2 regulate apoptosis is that activation of caspase3 by its DNA demethylation activity. Future studies could explore the specific genes or regulatory regions affected by TET2 mediated DNA demethylation and their functional implications in caspase3 activation and apoptosis.


A
DMSO
CSE
MG132
TET2
BCL2
CCP3
β-actin
260 kDa
26 kDa
19 kDa
17 kDa
42 kDa
B
protein relative expression
TET2
BCL2
CCP3
DMSO
MG132
CSE
MG132+CSE
C
DMSO
MG132
CSE
MG132+CSE
D
Apoptosis index(%)
E
CSE
shCtrl
shUSP21
MG132
TET2
USP21
BCL2
CCP3
β-actin
260 kDa
63 kDa
26 kDa
19 kDa
17 kDa
42 kDa
F
protein relative expression
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Apoptosis index(%)

Figure 6. Inhibition of TET2 protein degradation protects CSE-induced apoptosis in BEAS-2b cells

(A and B) Western blot of TET2, BCL2, cleaved caspase3(CCP3) and β-actin protein expression in the BEAS-2b cells. Densitometry analysis of protein expression.

(C and D) Flow cytometry for detecting cell apoptosis and apoptosis index between different groups.

(E and F) Western blot of TET2, USP21, BCL2, cleaved caspase3(CCP3) and β-actin protein expression in the BEAS-2b cells. Densitometry analysis of protein expression.

(G and H) Flow cytometry for detecting cell apoptosis and apoptosis index between different groups.

(I and J) Western blot of TET2, USP21, BCL2, cleaved caspase3(CCP3) and β-actin protein expression in the BEAS-2b cells.

(K and L) Flow cytometry for detecting cell apoptosis and apoptosis index between different groups. Densitometry analysis of protein expression. CSE: BEAS-2b cells exposed to CSE (5% CSE at 24h); DMSO: BEAS-2b cells exposed to DMSO; MG132: BEAS-2b cells exposed to MG132.shCtrl: BEAS-2b cells exposed to negative control; shUSP21: BEAS-2b cells exposed to shUSP21; TET2: BEAS-2b cells exposed to overexpression plasmid of TET2. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, compared between the marked groups.

To furthermore explore how CSE regulates TET2 expression to effect cell apoptosis. From the results in current study, we found that TET2 protein expression in COPD patients were obviously decreased, while mRNA levels were not significantly different. It seems that the effect of CS on TET2 expression is at the level of its stability, rather than its transcription. AS a possible mechanism, we predict that CSE may participate in the regulation of TET2 protein activity through protein post-translational modification. In our results, MG132, which is characteristic of blocking protein degradation, significantly boosted the ubiquitination of TET2 in BEAS-2b cells. In addition, CHX, which blocks protein translation, significantly reduced the half-life of TET2 in BEAS-2b cells. These data suggested that TET2 can be regulated by ubiquitin-mediated degradation.

Ubiquitin-specific peptidase is a subclass of deubiquitinating enzymes (DUBs) superfamily.[33] Previous study has been reported that USP49 was involved in the development of COPD.[28] Therefore, we focus on the alternation of USP family in COPD. Furthermore, we identified that the DUB USP21 can interact with TET2 to promote its stability via deubiquitination through bioinformatics analysis and *in vitro* cell experiments, including immunoprecipitation, immunofluorescence, and ubiquitination assay. TET2 can be regulated by ubiquitination. Published research has shown that TET2 can bind to VprBP and was ubiquitylated by the E3 ubiquitin ligase during development and in tumor suppression.[34] Another research also reported that DUB USP15 modulate TET2 ubiquitination and involved in tumor immunity.[35] In addition, USP21 can stabilize protein expression such as GATA3 and Gli by mediating their deubiquitination.[36,37] To explore weather TET2 attenuate CSE-induced apoptosis by its protein stability. We observed that knockdown the expression of USP21 can promote TET2 ubiquitination and degradation, and reduced the expression of anti-apoptotic protein BCL2 decreases, increased the level of pro-apoptotic protein cleaved caspase3 to promote cell apoptosis. Conversely, these alternations can be partially reversed by adding MG132 or overexpressing TET2. Collectively, our results indicate that the protect effect of TET2 in CSE-induced apoptosis by its protein stability.

Conclusions

In summary, we clarified that TET2 was downregulated in the lung tissue of CS exposed COPD animal model and COPD patient. TET2 knockdown can promote cell apoptosis by CSE treatment, while enhance the level of TET2 can attenuate CSE-induced apoptosis. Mechanistically, DUB USP21 interacted with TET2 and mediated its proteasomal degradation to regulate the apoptosis treated with CSE. The findings of the present study demonstrate that TET2 play a protective role in COPD pathogenesis. And this may provide some contribution to precise treatment of COPD in the future.

Limitations of the study

Although TET2 plays a critical role in the pathogenesis of COPD, especially in airway epithelial cell apoptosis, the specific molecular mechanism of TET2 on regulating apoptosis needs to be further explored.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109252.

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AUTHOR CONTRIBUTIONS

L.J.L., Z.H.Z., Y.L., and Y.C. conceived and designed the research; L.J.L., Z.H.Z., T.L., X.M.L., Y.N.C., and Y.G.T. performed experiments; L.J.L. and Z.H.Z. analyzed the data; L.J.L. and Z.H.Z. drafted the manuscript. All authors reviewed and approved the final version manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-TET2	Milipore	Cat: MABE462
Anti-BCL2	Cell Signaling Technology	Cat: #3498
Anti-cleaved caspase3	Cell Signaling Technology	Cat: #9664
Anti-USP21	Proteintech	Cat: 17856-1-AP
Anti-β-actin	Proteintech	Cat: 66009-1-Ig
Anti-Ub	Santa Cruz Biotechnology	sc-8017
Chemicals, peptides, and recombinant proteins		
Lipofectamine 3000	Thermo Fisher Scientific	Cat: #L3000008
Cycloheximide	Med Chem Express	HY-12320
MG132	Med Chem Express	HY-13259
Cigarette	Marlboro	6901028143431
TET2 overexpressed plasmids	Genechem	GOSE0219235
USP21 overexpressed plasmids	WZ Biosciences	CH805297
USP21 shRNA	Genechem	GIEE02923217
TET2 siRNA	RiboBio	P202110180092
Flag-tagged DUB overexpression plasmids	Professor Yongguang Tao, Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Department of Pathology, Xiangya Hospital, Central South University.	N/A
Critical commercial assays		
TUNEL BrightGreen Apoptosis Detection Kit	Vazyme	A112-03
FITC Annexin V Apoptosis Detection Kit	Beyotime	C1062L
Experimental models: Cell lines		
Beas-2b	Institute for advanced study, Central South University	N/A
Experimental models: Organisms/strains		
C57BL/6J mice	Slyke Jingda Laboratory	N/A
TET2 $^{-/-}$ mice	Academician Guoliang Xu, Laboratory of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yan Chen (chenyan99727@csu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human subjects

Pulmonary tissues were obtained from patients undergoing pneumonectomy at the Second Xiangya Hospital of Central South University between January 2017 and July 2019.The patients were divided into three groups: nonsmoker group (n = 4), smoker group (n = 4), and COPD group (stable stage, n = 5). The patients with COPD had airflow limitation (forced expiratory volume in 1 s/forced vital capacity [FEV1/FVC] <0.7). The ages of the three groups were 54.49 ± 9.23 in the nonsmoker group, 57.65 ± 7.18 in the smoker group and 61.28 ± 6.96 in the COPD group. The genders of the three groups were 1 male and 3 females in the nonsmoker group, 4 males in the smoker group and 5 males in the COPD group. The FEV/FVC of the three groups were 87.34 ± 17.19 in the nonsmoker group, 77.72 ± 9.12 in the smoker group and 54.51 ± 8.09 in the COPD group. And other information of relevant participants is available in Table S1. The study was approved by the Medical Ethics Committee of the Second Xiangya Hospital of Central South University (protocol No. 2018010). Written informed consent was obtained from all human subjects before their enrollment into the study, which was conducted in accordance with the Declaration of Helsinki.

Animal

C57BL/6J mice as wild-type (WT) mice (6–8 weeks, male, Slyke Jingda Laboratory Animal Co., Ltd., Hunan, China) and TET2$^{-/-}$ mice were generous gifts from Academician Guoliang Xu, Laboratory of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. Breeding and genotyping of TET2 deficient mice were generated as previously described.[38] age-and sex-matched mice (6–8 weeks, male) were divided into four groups by a free online randomization tool (https://www.graphpad.com/quickcalcs/randomize1.cfm): WT + Air group, WT + CS group, TET2$^{-/-}$ + Air group, TET2$^{-/-}$ + CS group. CS exposure according to the protocol of previous study.[39,40] Briefly, WT and TET2$^{-/-}$mice were exposed to the smoke of cigarettes using a clear plastic box twice daily with 90 min smoke-free intervals, 5 days a week for 24 weeks. Control mice were exposed to room air only. The animals were sacrificed at the end of the modeling period. The left lung tissues were then collected for histopathology analysis and right lung tissues were stored in −80°C for western blot analysis. All *in vivo* manipulations were approved by the Medical Ethics Committee of Second Xiangya Hospital of Central South University (protocol No. 2018010).

Cell

Airway epithelial cell BEAS-2b was purchased from the Institute for advanced study, Central South University (Changsha, China) and cultured in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum and 50U/mL penicillin and streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO2 culture chamber. Starvation for 24h was performed before exposure to CS extract (CSE), siRNA, and/or plasmids.

METHOD DETAILS

Lung tissue morphometry and immunohistochemistry (IHC)

Lung tissue samples were stained with hematoxylin-eosin (HE) staining and the morphology change of lung tissues was observed by light microscopy. The mean linear intercept (MLI) and destructive index (DI) were measured at a magnification of 100×. MLI was obtained by dividing the length of a line drawn on a lung section by the total number of alveolar intervals that pass through that line. DI was the percentage of destroyed alveoli divided by the total number of alveoli counted in the same lung section.[41] About IHC, the slides were incubated with TET2 antibody. Quantitative measurements of TET2 expression by counting integrated optical density (IOD) in the lung tissue.[42]

TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed according to apoptosis detection kit (A112-03, Vazyme Biotechnology Co., Ltd, Nanjing, China). We randomly selected areas to measure the apoptotic index (AI). Fields containing non-parenchymal structures such as large airways or vessels were excluded. TUNEL-positive cells were counted, and the apoptotic index was calculated as a ratio of (apoptotic cell number)/(total cell number) in each field under magnifying view of 200×.

Reagents

TET2 antibody (Cat: MABE462) was form Milipore company (Massachusetts, USA), β-actin antibody (Cat:66009-1-Ig) and USP21(Cat:17856-1-AP) were from proteintech (Rosemont, USA). BCL2 antibody (Cat: #3498) and cleaved caspase3 antibody (Cat: #9664) are from CST company (Massachusetts, USA). Lipofectamine 3000 (Cat: #L3000008) were purchased from Thermo Fisher Scientific (Waltham, MA, USA), Cycloheximide (CHX, HY-12320) and MG132 (HY-13259) were purchased from Med Chem Express (New Jersey, USA).

PREPARATION OF CSE

One cigarette (Marlboro; Longyan Tobacco Industrial Co, Ltd, Fujian, China; tar: 10 mg, nicotine: 1.1 mg, carbon monoxide: 11 mg) was burned and the smoke was collected in a vessel containing DMEM (10 mL) defined as 100% CSE. The smoke was filtered through a 0.2-μm pore-size filter and the pH of the solution was 7.2–7.4. CSE was prepared freshly in each intervention.[43]

Plasmids and siRNA interference

Flag-tagged DUB overexpression plasmids were a gift from Professor Yongguang Tao, Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Department of Pathology, Xiangya Hospital, Central South University, Changsha, China. The plasmid vector GV362(GOSE0219235), TET2 overexpressed plasmids (GOSE0219235) and USP21 shRNA (GIEE02923217) were purchased from Genechem Biotechnology Co., LTD (Shanghai, China). USP21 overexpressed plasmids (CH805297) was purchased from WZ Biosciences Inc (Shandong, China). Scramble siRNA, TET2 siRNA (target sequence: GTAGCAGTGGAGAGCTACA) were purchased from RiboBio company (P202110180092, Guangzhou, China). BEAS-2b cells were transfected with different plasmid or others using LipoMaxTM (ECS000165, Sudgen, China) according to the manufacturer's instructions.

Western blot analysis

Protein levels were determined using western blot. Proteins were resolved in SDS-polyacrylamide electrophoresis gels and transferred to PVDF membrane, which were then blocked and incubated with antibodies: TET2, BCL2, cleaved caspase3, USP21, Ub, β-actin. After incubation with HRP-conjugated goat anti-mouse/rabbit IgG antibody, the immunoreactive bands were visualized by using the enhanced chemiluminescence (ECL plus; GE Healthcare, Buckingham, UK). Results are expressed as relative densities.

Isolation of RNA and qPCR

Total RNA was extracted from the samples using TRIZOL reagent (15596026, Invitrogen, California, USA) following manufacturer's instructions. Isolated mRNA (1 μg each) was reverse transcribed into cDNA using the Reverse transcription system in the presence of oligo dT primers (K16225, Thermo Scientific, Massachusetts, USA). qPCR was carried out in a 20 μL reaction system using SYBR Green One-Step qPCR system (Yeasen Biotechnology, Shanghai, China). GAPDH was used as an internal control. The sequences of all primers used were:

Human-TET2-F: 5′-GCTTACCGAGACGCTGAGGAAA-3′.
Human-TET2-R: 5′-AGAGAAGGAGGCACCACAGGTT-3′.
Mouse-TET2-F: 5′-ACCTGGCTACTGTCATTGCTCC-3′.
Mouse-TET2-R: 5′- TGCAGTGACTCCTGAGAATGGC-3′.
Human-GAPDH-F: 5′-GTCTCCTCTGACTTCAACAGCG-3′.
Human-GAPDH-R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′.
Mouse-GAPDH-F: 5′-CATCACTGCCACCCAGAAGACTG-3′.
Mouse-GAPDH-R: 5′-ATGCCAGTGAGCTTCCCGTTCAG-3′.

Bioinformatics analysis

Briefly, using GEO Profiles of the GEO public database to search each ubiquitin molecule and COPD in turn (https://www.ncbi.nlm.nih.gov/geoprofiles).Then download the expression data of each molecule in smokers' epithelial cells, and the obtained data are normalized by Z-scan. $Z= (X-\mu)/\sigma$, Where, X represents standardized random variable, μ represents sample mean, σ represents sample standard deviation.

Immunoprecipitation

In brief, cell lysate was incubated overnight with TET2 and USP21 antibodies for overnight at 4°C. Protein A/G PLUS-Agarose were added and incubated for 2h at 4°C. Next, the mixture was eluted and investigated for the expression of associated markers by western blot.[44]

Ubiquitination assay

BEAS-2b cell lines were transduced with USP21 shRNA to establish stable knockdown cell lines. After treatment with 10 μM MG132 for 6 h, cells were lysed directly in 10 mM Tris-HCl buffer containing 2% SDS and boiled. The cleared lysates were quantified, and an equal amount of each lysate was used for immunoprecipitation with protein A/G agarose pre-bound with the Ub antibody (Santa Cruz Biotechnology, sc-8017). The resin beads were washed with lysis buffer, and samples were eluted. The eluted fraction was further verified by western blot.

Immunofluorescence staining assays

BEAS-2B cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After being incubated with the indicated TET2 and USP21 antibodies overnight at 4°C followed by a secondary fluorescent antibody for 1 h at 37°C in the dark. The nucleus was stained with 4′6-diamidino-2-phenylindole (DAPI). Images were visualized with an inverted fluorescence microscope.

Flow cytometry analysis

The flow cytometry was operated according to the instruction of the FITC Annexin V Apoptosis Detection Kit (Beyotime BioTECH, Shanghai, China). BEAS-2B Cells with different treatment were collected using trypsin without EDTA and washed three times with PBS followed by the FITC for 15min and PI for 5 min at room temperature, and finally examined by the flow cytometry.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were presented as means ± SD. Two-group comparisons were performed using an independent sample t-test. One-way ANOVA was used for multiple comparisons. The SPSS 23.0 was used to analysis the data and $p < 0.05$ was considered statistically significant.