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Nat Med. Author manuscript; available in PMC 2014 March 17.

Published in final edited form as:

Nat Med. 2010 July ; 16(7): 767–773. doi:10.1038/nm.2157.

Rtp801, a suppressor of mTOR signaling, is an essential mediator of cigarette smoke – induced pulmonary injury and emphysema

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Abstract

Rtp801, a stress – related protein triggered by adverse environmental conditions, inhibits mTOR and enhances oxidative stress – dependent cell death. We postulated that *Rtp801* acts as potential amplifying switch in the development of cigarette smoke – induced lung injury, leading to emphysema. *Rtp801* was overexpressed in human emphysematous lungs and in lungs of mice exposed to cigarette smoke. The upregulation of *Rtp801* expression by cigarette smoke in the lung relied on oxidative stress – dependent activation of the CCAAT response element. *Rtp801* was necessary and sufficient for NF – κ B activation in cultured cells and, when forcefully expressed

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Authors' contributions:

TY, EF, and RMT designed the experiments, analyzed the data, and composed the manuscript; IM, AKB, JB, MP, LZ, AG, UC, TM, AR, EB HA, NN, AG, and IP performed several of the in vitro and in vivo experiments; RKT, TR, TS and SB provided mouse lung samples for *Rtp801* expression studies; CG provided normal human lung samples for *Rtp801* expression studies; MM and SD performed the chronic exposure to cigarette smoke.

in mouse lungs, it promoted NF – kB activation, alveolar inflammation, oxidative stress, and apoptosis of alveolar septal cells. On the other hand, *Rtp801* $^{-/-}$ mice were markedly protected against acute cigarette smoke – induced lung injury, partly via increased mTOR signaling, and, when exposed chronically, against emphysema. Our data support the notion that *Rtp801* may represent an important molecular sensor and mediator of lung injury to cigarette smoke.

Keywords

Rtp801; cigarette smoke; oxidative stress; apoptosis; inflammation; NF –κB; rapamycin

Introduction

Emphysematous lung destruction is a major component of chronic obstructive pulmonary disease (COPD), a highly prevalent and morbid disease predominantly caused by exposure to cigarette smoke (CSk) and environmental pollutants 1. Lung inflammation and excessive extracellular matrix proteolysis have been implicated as mediators of lung injury by CSk. CSk – triggered oxidative stress initiates lung inflammation and progressively disrupts cellular signaling involved in maintenance of lung integrity, eventually leading to organ aging and cell senescence 2. Amplification of these processes by oxidants generated by inflammatory and parenchymal cells may enhance apoptotic septal destruction 3 and cellular autophagy 4, involving the action of endogenous mediators, such as ceramide 5–7.

Rtp801 8, also known as *Redd1* (for *re*gulated in *d*evelopment and *D*NA *d*amage responses) 9, was identified based on its induction by hypoxia 8 or DNA damage 9. Brain ischemia leads to upregulated neuronal *Rtp801* expression 8 and induction of *RTP801* in the retinal inner nuclear cell layer is required for neural retina and central vessel endothelial cell death in mice with retinopathy of prematurity 10. Moreover, overexpression of *Rtp801* causes apoptosis of cultured cells and lung cells *in vivo* 8, leading to enhanced oxidative stress 9. Rtp801 inhibits cell growth and protein synthesis directed by the mammalian target of rapamycin (mTOR) 11, 12 via activation of Tsc – 2 (tuberin), a negative regulator of mTOR 11, 13, 14. Decreased mTOR activity downregulates hypoxia inducible factor (HIF) – 1 – dependent vascular endothelial growth factor (VEGF), which has been linked to experimental and human emphysema 1, 15, 16.

In the present study, we addressed whether the axis *Rtp801*/mTOR might integrate environmental stresses due to CSk with the activation of inflammation and cell death signals, leading to lung tissue damage and emphysematous destruction.

Results

Rtp801 expression in human lung emphysema and in cigarette smoke – exposed mouse lungs

We found a significantly upregulated *Rtp801* expression in lungs of patients with advanced emphysema compared with normal lungs (Fig. 1a), notably in alveolar septa of lungs with advanced emphysema when compared with normal lungs (Fig. 1b). Lungs of healthy smokers and patients with mild to moderate COPD had increased expression of *Rtp801*

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mRNA levels, while lungs of patients with severe disease expressed similar levels of *Rtp801* transcripts as normal non smoker's lungs. (Fig. 1c and Supplementary Table 1). These findings suggest that Rtp801 may undergo posttranscriptional stabilization in lungs with advanced COPD, as recently shown with cultured cells exposed to hypoxia 17.

We tested whether *Rtp801* expression may be upregulated by CSk – induced lung oxidative stress 18. Mice exposed to CSk for up to 7 days showed increased lung expression of *Rtp801* in alveolar septa by immunohistochemistry (IHC) (Fig. 2a) and Western blot analyses (Fig. 2b). Alveolar type II pneumocytes showed the highest levels of Rtp801, followed by that of endothelial cells and minimal expression in type I pneumocytes (Fig. 2c). Of note, expression of Rtp801 appeared to predominate in alveolar septal cells rather than inflammatory cells based on the more modest expression of *Rtp801* mRNA (Fig. 2d) and protein levels (data not shown) in cells obtained by bronchoalveolar lavage (Bal) (composed predominantly by inflammatory cells) and lack of the more sensitive IHC signal in macrophages. Mice exposed to CSk for 4 – 6 months also exhibited increased *Rtp801* expression levels (Supplementary Fig. 1a) 19, 20.

Role of oxidative stress in cigarette smoke – induced *Rtp801* expression

Consistent with the prior observation that oxidative stress induces *Rtp801* expression *in vitro* 8, 21, the antioxidant *N* – Acetyl – L – Cysteine (NAC, 10mM) completely prevented lung Rtp801 up regulation caused by CSk exposure for 1 day (Fig. 2e). Consistent with these results, lungs of mice lacking the nuclear erythroid-related factor (*Nrf) – 2*, with heightened sensitivity to CSk – induced lung injury 19, showed increased *Rtp801* mRNA signal when compared with wildtype littermates chronically exposed to CSk (Supplementary Fig. 1a). However, the resistant strain CD – 1 and the more sensitive strain C57Bl6/J had similar levels of *Rtp801* mRNA when exposed to CSk for 1 day (Fig. 2 and data not shown).

The *Rtp801* promoter contains regulatory elements that bind Elk, CCAAT/enhancer binding protein (C/EBP), HNF – 4, NF – κB, p53, and HIF – 1α transcription factors 21. The CSk induction of reporter plasmids 21 driven by the *Rtp801* 2.5 kB promoter 21 involved the activation of the CCAAT motif as a critical point mutation in this response element reduced CSk – dependent luciferase activity to baseline levels in mouse lung fibroblasts (MLF) (Fig. 2f), human bronchial epithelial cell line Beas2B (Supplementary Fig. 1b), and human fetal kidney epithelial line HEK293 cells (data not shown). Enhanced expression of endogenous Rtp801 in duplicate cell samples paralleled the induction of the *Rtp801 – promoter* (Fig. 2g and Supplementary Fig. 1c). NAC added with CSE prevented the activation of the *Rtp801* promoter reporter construct in MLF (Fig. 2f).

Rtp801 overexpression and activation of NF – kB due to cigarette smoke

CSk – associated oxidative stress 22 may promote lung inflammation through NF – κB signaling 23. Accordingly, inhibition of NF – κB activation in lungs infected with adenovirus – IκBα super repressor 24 significantly ($P<0.05$) decreased lipopolysacharide (LPS – positive control) – and, notably, CSk – induced accumulation of inflammatory cells in the Bal (Fig. 3a and Supplementary Figs. 2a – c). However, expression of the IκBα super repressor had no effect on CSk – dependent induction of the oxidative stress markers 3 –

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nitrotyrosine (Supplementary Fig. 2d) and 8 – oxo – 2 deoxy – guanosine (data not shown) as compared with CSk – exposed control mice. The up regulation of *Rtp801* mRNA levels driven by acute CSk exposure was not affected by expression of the IκB super repressor (Supplementary Fig. 2e). LPS, an activator of NF – κB, did not alter *Rtp801* gene expression in challenged lungs when compared with PBS – instilled lungs, and, conversely, the IκB super repressor did not affect lung *Rtp801* mRNA levels after treatment by LPS (Supplementary Fig. 2f) or in cultured A549 cells treated with CSE or TNF-α (Supplementary Figs. 2g,h). These data positioned the stress – dependent up regulation of *Rtp801* by CSk upstream of NF – κB activation both *in vivo* and *in vitro*.

Human *RTP801* overexpression sufficed to activate NF – κB in rat lung microvascular endothelial cells to levels similar to those elicited by LPS in the same cells (Fig. 3b). Furthermore, Rtp801 was not only sufficient but also required for NF – κB activation since *Rtp801* $^{-/-}$ MLF did not activate the NF – κB dependent promoter when stimulated with CSE or LPS (Fig. 3c).

In ambient room air (RA) conditions, *Rtp801* $^{-/-}$ lungs showed a trend towards enhanced baseline Serine (Ser) 536 phosphorylation in p65 NF – κB (which closely correlates with NF – κB transcriptional activation 25). However, following exposure to CSk, only wildtype mice demonstrated a sizable NF – κB response with phosphorylation of p65 on Ser 536, whereas this response was abolished in *Rtp801* $^{-/-}$ mice (Fig. 3d); MLF displayed similar CSE – induced NF – κB responses (Supplementary Fig. 3a). We also detected increased mRNA expression of the NF – κB – dependent chemokine macrophage inflammatory protein (MIP) – 2α (CXCL – 2) 26 in lungs from wildtype mice exposed to CSk, compared with similarly treated *Rtp801* $^{-/-}$ mice (Supplementary Fig. 3b). The stress – and CSk – inducible p38MAPK was probably not involved induced NF – κB activation by CSk as the p38MAPK inhibitor SB239063 did not reduce levels of phosphorylation of p65 NF – κB in Ser536 or in Ser276 in mice exposed to CSk (Supplementary Figs. 3c–e).

Further supporting the observed link between oxidative stress and NF – κB activation26, *Rtp801* $^{-/-}$ MEF contained lower levels of reactive oxygen species (ROS) than wildtype cells, either at baseline or following treatment with H_2O_2 from 6 to 24 h (Fig. 3e and Supplementary Fig. 3f). Likewise, the mouse lung epithelial cell line (MLE) – 15 27 showed decreased oxidative stress caused by H_2O_2 after transduction of a specific siRNA lowered *Rtp801* mRNA levels by approximately 50% as compared with Rtp801 – replete cells treated with a scrambled siRNA (Supplementary Fig. 3g). These findings are in line with higher expression of the inducible anti – oxidant gene *heme oxygenase* (HO) – 1 mRNA in CSk – exposed *Rtp801* $^{-/-}$ lungs compared with wildtype controls (see below and Supplementary Fig. 3).

Rtp801 overexpression in mouse lungs and alveolar injury

We addressed next whether forced overexpression of *Rtp801* in mouse lungs elicits features similar to those caused by CSk exposure. Intratracheally – instilled human *RTP801* cDNA led to increased *Rtp801* expression, localized predominantly in alveolar type II cells rather than small airway cells (Fig. 4a), when compared with lungs instilled with the loss – of – function mutant *Rtp801 – RPAA* (which is unable to remove the inhibitory protein 14 – 3 – 3

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from Tsc – 2 and consequently block TORC1 activity 28) or the empty vector pcDNA3. Up regulation of *RTP801* triggered lung inflammation, oxidative stress, and alveolar cell death, (Figs. 4b – d and Supplementary Fig. 4a).

Intra – tracheal infection of wildtype mice with adenoassociated – virus serotype 5 (AAV5) – *RTP801* (AAV5 infects lung epithelial cells for prolonged periods of time without causing inflammation 29) for 4 weeks augmented the numbers of macrophages in airspaces compared with negative control AAV – *Cre* transduced lungs (Fig. 4e). However, we did not observe significant (P>0.05) airspace enlargement, which is often seen with longer (i.e., 6 months) exposures to Csk (data not shown).

Rtp801 and cigarette smoke – induced acute and chronic pulmonary alveolar injury

Rtp801 $^{-/-}$ mice have normal lung structure at 1, 2, 4, and 12 weeks when compared with wildtype littermates, based on analyses of lung sections stained with hematoxylin eosin, elastic, type II cell pro surfactant protein C expression, and terminal airway epithelial Clara cell antigen IHC (data not shown). Following the demonstration that forced *RTP801* expression in mouse lungs resulted in oxidative stress, apoptosis, and inflammation, we next assessed whether *Rtp801* expression is necessary for CSk-induced lung pathology. *Rtp801* $^{-/-}$ mice, when exposed to CSk for up to 7 days, showed complete protection against acute inflammation, including reduced numbers of total cells (Fig. 5a), macrophages (Fig. 5b), and neutrophils (Fig. 5c) in Bal fluid and reduced neutrophil influx in lung parenchyma (Fig. 5d) when compared with similarly treated wildtype mice. *Rtp801* $^{-/-}$ mice also displayed significantly reduced (P<0.05) numbers of apoptotic cells as assessed by IHC staining of activated caspase 3 (Fig. 5e) and poly – ADP ribose polymerase (PARP) cleavage in immunoblots (Supplementary Fig. 4b). Furthermore, when exposed to CSk, *Rtp801* $^{-/-}$ mice had increased expression of Hif – 1α – dependent lung protective genes, such as $VEGF_{120}$, and the *glucose transporter* (*GLUT*) – *1* mRNA (Supplementary Figs. 3i – j).

Consistent with protection against pathology caused by short – term CSk exposure, *Rtp801* $^{-/-}$ lungs had preserved alveolar structure after 6 months of smoking 30, with no increases in mean linear intercepts (vs. RA – exposed mice) and sparse accumulation of intraalveolar macrophages. On the other hand, wildtype mice exhibited the classic morphologic features of airspace enlargement and accumulation of alveolar macrophages filled with smoking pigment 30 (Figs. 5f,g). These findings were accompanied by decreased expression and nuclear accumulation of NF – κB in *Rtp801* $^{-/-}$ when compared with wildtype lungs (Supplementary Fig. 4c). These results indicate that *Rtp801* might have a critical role in the pathogenesis of experimental CSk – induced pulmonary injury and emphysema.

mTOR and protection of *Rtp801* $^{-/-}$ mice against cigarette smoke – induced injury

Rtp801 negatively regulates mTOR activity, via dissociation of the inhibitory protein 14 – 3 – 3 from Tsc – 2, leading to its blockade of mTOR11, 28, evidenced by decreased phosphorylation of S6 Kinase, its phosphorylation target S6, and 4EBP1 31. Accordingly, we found enhanced S6 phosphorylation on Ser235/236 (p-S6) in *Rtp801* $^{-/-}$ RA – exposed

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lungs and MLF compared with wildtype lungs and MLF, respectively (Fig. 6a and Supplementary Figs. 5a,b). The selective mTORC1 inhibitor rapamycin pronouncedly blocked p-S6 in mouse lungs and MLF under control conditions (Fig. 6a and Supplementary Fig. 5b).

To further define the potential function of mTOR in lung homeostasis in RA and in response to CSk, we investigated the influence of rapamycin on pulmonary inflammation and apoptosis in wildtype mice in RA and in wildtype and mice after exposure to CSk. CSk led to a marked increase in p-S6 in wildtype lungs (close to levels in *Rtp801* $^{-/-}$ lungs) on day 1 when compared with RA (Supplementary Fig. 5a). In RA – kept wildtype mice, rapamycin led to increased inflammatory cells in Bal (Figs. 6 b,c) with lung enhanced expression of phosphorylated Ser536 p65 NFκB (Fig. 6d) and heightened numbers of apoptotic alveolar cells in lung parenchyma (Fig. 6e). Furthermore, rapamycin pretreatment partially abrogated the protection against CSk – induced inflammation observed in the *Rtp801* $^{-/-}$ mice. Paradoxically, rapamycin decreased alveolar inflammation and phosphorylated Ser536 p65 NF– κB levels in wildtype mice exposed to CSk (Figs. 6 f,g). The aggregate of these findings indicates that mTOR activity contributes to maintenance of lung homeostasis under normal conditions and partly accounts for the resistance to CSk – induced alveolar inflammation in *Rtp801* $^{-/-}$ mice.

Discussion

Our findings indicate that *Rtp801* plays a critical role in the pathogenesis of alveolar injury caused by CSk, amplifying inflammatory and cell death responses by regulating negatively mTOR signaling and activating NF – κB. Enhanced NF – κB activation *in vitro* and particularly *in vivo* leads to expression of cytokines (e.g., MIP-2α 32) responsible for the recruitment of neutrophils and macrophages 33. Moreover, *RTP801* also directs alveolar cell apoptosis due to acute CSk, which might further promote lung inflammation, oxidative stress, and extracellular matrix degradation 3. In rodents, acute CSk causes fragmentation of lung elastin 34, generating chemotactic elastin peptides 5, and a collagen – degradation product, the tripeptide, proline – glycin – proline (PGP), which binds to CXCR – 2 and stimulates neutrophil lung infiltration and alveolar destruction 7. Alveolar injury caused by acute CSk exposures might therefore have relevance to chronic emphysematous lung destruction. Our finding that type II and endothelial cells (rather than inflammatory cells) up regulate *Rtp801* in response to CSk underscore their critical role in the pathogenesis of alveolar destruction in emphysema and cell specific responses that drive lung pathology in COPD. This central role of alveolar septal cells has been supported by models of emphysema caused by lung epithelial cell overexpression of cytokines 35 and by targeted apoptosis of lung capillary endothelial cells 36.

Oxidative stress is a potential key mechanism that links CSk with lung inflammation and tissue injury, including alveolar septal cell apoptosis 37. Activation of *Rtp801* expression relies on oxidative stress and *Rtp801* $^{-/-}$ enhances oxidative stress when overexpressed 8, 38. These unique dual properties position Rtp801 as a potential key amplifier of ROS in acute and chronic pathological conditions and determinant of tissue injury caused by CSk. Indeed, in the present study, Rtp801 overexpression in lung epithelial cells *in vivo* not only

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promoted septal cell apoptosis 8, but also enhanced lung macrophage infiltration. These properties may promote aseptic innate inflammation 39 and therefore contribute to persistence of inflammation in COPD despite smoking cessation, via stimulation of auto – immunity 40, 41. Moreover, *Rtp801* appears not to directly affect Nrf – 2 signaling based on gene expression profiling of wildtype vs. *Rtp801* $^{-/-}$ lungs exposed to CSk (Yoshida et al, unpublished observations). Of note, our results indicate that *Rtp801* is both required and sufficient for the activation of NF – κB *in vitro* and *in vivo* by both CSk and LPS (no general abnormalities were seen in peripheral blood cells in *Rtp801* $^{-/-}$ mice (EF, unpublished observation)), allowing for further amplification of oxidant generation by inflammatory and parenchymal cells 42. The finding that the mutant *Rtp801 – RPPA*, which does not activate Tsc – 2, did not increase lung injury when acutely overexpressed implies that inhibition of mTOR signaling may be mechanistically involved in CSk – triggered proinflammatory and apoptotic effects of *Rtp801* 43. *Rtp801* may therefore link environmental stresses, the ensuing oxidative stress, with the activation of innate immunity via downregulation of mTOR signaling.

Consistent with the inhibitory role of *RTP801* in mTOR signaling, the CSk – resistant *Rtp801* $^{-/-}$ mice had increased lung mTOR signaling under normal conditions when compared with susceptible wildtype lungs. Of note, we also found that, when compared with wildtype mice, *Rtp801* $^{-/-}$lungs express higher levels of lung protective genes including $VEGF_{121}$ and the inducible HO – 1, which produces the antioxidant and anti – inflammatory metabolites, i.e., carbon monoxide, biliverdin – IXα/bilirubin, and ferrous iron 44, 45. The protective role of enhanced mTOR in *Rtp801* $^{-/-}$ lungs against acute CSk was disrupted by rapamycin, leading to alveolar inflammation and NF – κB phosphorylation in CSk – exposed mice. These results and the observed alveolar injury in wildtype mice by rapamycin in RA are in line with the described actions of rapamycin in promoting inflammation or apoptosis 46–48. However, mTOR is a critical signaling pathway not only in lung parenchymal cells but also in infiltrating inflammatory cells, possibly playing cell – and injury – context specific roles, which might lead to opposing effects in models of disease. The cell – specific (i.e., alveolar vs. inflammatory cells) regulation of *RTP801* may explain the finding of increased p – S6 expression in wildtype lungs exposed to CSk. In contrast to the effects in *Rtp801* $^{-/-}$ mice, rapamycin protected against acute CSk inflammation in *Rtp801* wildtype mice. This beneficial effect of rapamycin may be due to blockade of mTOR activation in inflammatory cells by CSk – induced AKT phosphorylation (49 and Yoshida et al, unpublished observations) or IKKβ activation 50. These opposing roles of mTOR are supported by the observations that rapamycin causes lymphocytic pneumonitis in transplant recipients 51, while it protects against experimental asthma 52. The timing and lung cell targets of mTOR inhibition might therefore be critical to define its beneficial vs. pathological roles in disease (Supplementary Fig. 6).

In conclusion, our data provide a novel insight into the role of *Rtp801* in stress – response sensing as an integrator of CSK – induced oxidative stress, NF – κB activation, and alveolar cell apoptosis, ultimately involved in the pathogenesis of emphysema. Genes, such as *Rtp801*, which are involved in environmental stress responses, particularly those converging

in mTOR, may provide first – tier tissue responses prior to the action of inflammation, extracellular matrix proteolysis, or triggers of apoptosis.

Methods

Antibodies and reagents are outlined in the Supplementary Methods.

Human lungs

Human normal and diseased lung tissues were obtained, processed, and assessed histologically as previously described 6. Lung samples for RT – PCR were obtained from National Jewish Health (normals and smokers) and from the Lung Tissue Repository Consortium (Supplementary Table 1). The experimental protocol was approved by the Western Institutional Review Board in lieu of the Johns Hopkins University and the Colorado Human Subject Review Boards.

Animals

Male *Rtp801* wildtype (C57BL/6 × 129SvEv F1) (Taconic) and *Rtp801* $^{-/-}$ mice (C57BL/6 × 129SvEv, 2 to 6 months – old) (generated by Lexicon Genetics Inc. for Quark Pharmaceuticals Inc.) were used 10. All experiments conducted in mice were approved by the Animal Care Use Committees of The Johns Hopkins University, Harvard University, and University of Colorado.

Cigarette smoke exposure

Mice were divided into RA – exposed control or exposed to CSk ($n = 3 – 7$ mice in each group) as described 19. The antioxidant NAC (500 mg/Kg body weight) was intraperitoneally injected to wildtype mice twice (day 0 and 1) before initiation of CSk exposure for 1 day and mice were sacrificed just after smoking. Six months exposure to CSk was performed as described 30. *Rtp801* expression was also investigated in lung samples from C57Bl/6 wildtype and *Nrf – 2* $^{-/-}$ mice exposed to 4 – 6 months CSk 20.

RTP801 $^{-/-}$ cDNA and AAV – *RTP801* constructs and administration to mice

Human *RTP801* and *Cre* cDNAs were cloned into an intermediate vector flanked by AAV internal inverted repeats. Expression was confirmed by Western blot analysis of transfected HEK293 cells. AAV5 – *hRTP801* and AAV5 – *Cre* viruses were prepared as previously described 53. *Rtp801* wildtype mice received 2×10^{10} of AAV5 – *hRTP801* or AAV5 – *Cre* viral particles via intratracheal instillation.

IκBα super repressor experiments

In vivo: *RTP801* wildtype (C57BL/6 × 129SvEv or C57Bl6/J mice as LPS controls; 2 month – old) were instilled intratracheally with 1×10^9 plaque forming units of Adenovirus – 5 (Ad 5) IκB super repressor or Ad5 LacZ (in 50 μL of sterile PBS). Seven to ten days later, the mice were exposed to CSk. Controls consisted of RA – exposed mice. LPS was instilled in 50 μl sterile PBS (30 ug ml^{-1}) and euthanized 16 h later. ***In vitro***: A549 cells were infected with an adenovirus construct containing IκB super repressor (S32/36A) or lacZ at approximately 10 pfu per cell. Twenty four – 36 h later, 2 and 5% CSE for 6 h (defined in

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preliminary studies with concentrations varying from 1 to 10% for 6, 12, 18 and 24 h) and TNF-α (10 ng ml^{-1}) for 1 hr as positive control were added.

Rapamycin experiments

In vivo: We used a protocol developed for rapamycin – induced blockade of experimental transplant rejection based on intraperitoneal administration 54, 55 or intratracheal instillation of rapamycin (1 mg/kg) or vehicle (1.8% DMSO) to wildtype and *Rtp801* $^{-/-}$ mice for 7 days prior to exposure to Csk for 4 days or RA. ***In vitro***: MLF were prepared as described and were pretreated with 10 nM of rapamycin for 3 days prior to CSE exposure, and then treated with 1% CSE for the indicated period.

Cell culture and cigarette smoke extract preparation

MLF and MEF were isolated from *Rtp801* wildtype and *Rtp801* $^{-/-}$ mice by collagenase digestion and were grown as outlined in the Supplementary Methods. Beas2B cells were cultured with bronchial epithelial basal supplement. MLFs and MEFs were used between the 3rd and 7th passages seven, while RLMEC and Beas2B were used up to passage 20. CSE was prepared as described previously 6.

Rtp801 and NF – κB promoter assays

For *Rtp801* promoter assay, MLF, HEK293, and Beas2B were transfected with firefly luciferase expression vectors of the full – length *Rtp801* 2.5 kb promoter or with a point mutation within C/EBP binding site or with an empty vector (pGL3 basic) 21. Two independent experiments, were performed each in triplicate (the standard deviation for each individual measurement within the triplicates was up to 10% of the mean). Parallel cell lysates samples were prepared for Rtp801 Western blot analysis.

For *NF – kB* promoter assay, approximately 8~11 × 10^4 MLFs and rat lung microvascular endothelial cells were transfected with pHTS *NF – kB* reporter vector encoding the *Firefly* luciferase gene and/or hRTP801 (779 bp) plasmid DNA or pcDNA3.1 (+). Transfection conditions are detailed in the Supplementary Methods.

Bronchoalveolar lavage and alveolar morphometry 19, 30, immunohistochemistry and immunofluorescence 19, 56, Western blotting, Determination of intracellular ROS production and cell death assay, isolation of RNA and quantitative RT – PCR are detailed in the Supplementary Methods.

Statistical analysis

Data are represented mean ± SEM. Multiple comparison analyses were done by ANOVA with Tukey's posthoc test or Kruskal – Wallis non – parametric ANOVA with Dunnet's T3 posthoc test, or Student's *t* – test or Wilcoxon rank – sum test, when involving two groups. Horizontal lines represent significant statistical ($P < 0.05$) comparisons among the listed (x – axis) experimental groups.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr T. Stevens (Univ. of South Alabama) for providing the primary rat lung microvascular endothelial cells, Dr. J. Whitsett (Univ. of Cincinnati) for allowing us to use the MLE – 15 cells, Dr. T. Flotte and Mr. P. Cruz (Univ. of Florida and Massachusetts) for preparing the AAV5 vectors, Dr. Y. Chen (Univ. of Indiana) for the *Rtp801* promoter constructs, Dr. I. Rahman (Univ. of Rochester) for sharing cigarette smoke – sensitive and resistant mouse lung specimens, Dr. D. Brenner (Univ. of California, San Diego) for the adenovirus IκB super repressor, Dr. L. Ellison (Dana Farber Cancer Center, Boston) for providing the *Rtp801* – RPAA construct, and Dr. S. Reynolds (National Jewish Health) for the anti – mouse CC10 antibody. This work was supported by the grants R01 NHLBI 66554, Alpha 1 Foundation, Flight Attendant Medical Research Institute (FAMRI), and Quark Pharmaceuticals (to RMT), R01 NHLBI 077328 (IP), COPD SCCOR P01 – NHLBI 085609, Project 1 (to RMT and IP) and Project 2 (to SB), RO1 NHLBI RO1 081205 (SB), and NHLBI R01 HL082541 – 01 (SDS). Anonymous, non – transplanted, non – diseased lung samples were obtained from Tissue Transformation Technologies, Inc., Edison, NJ.

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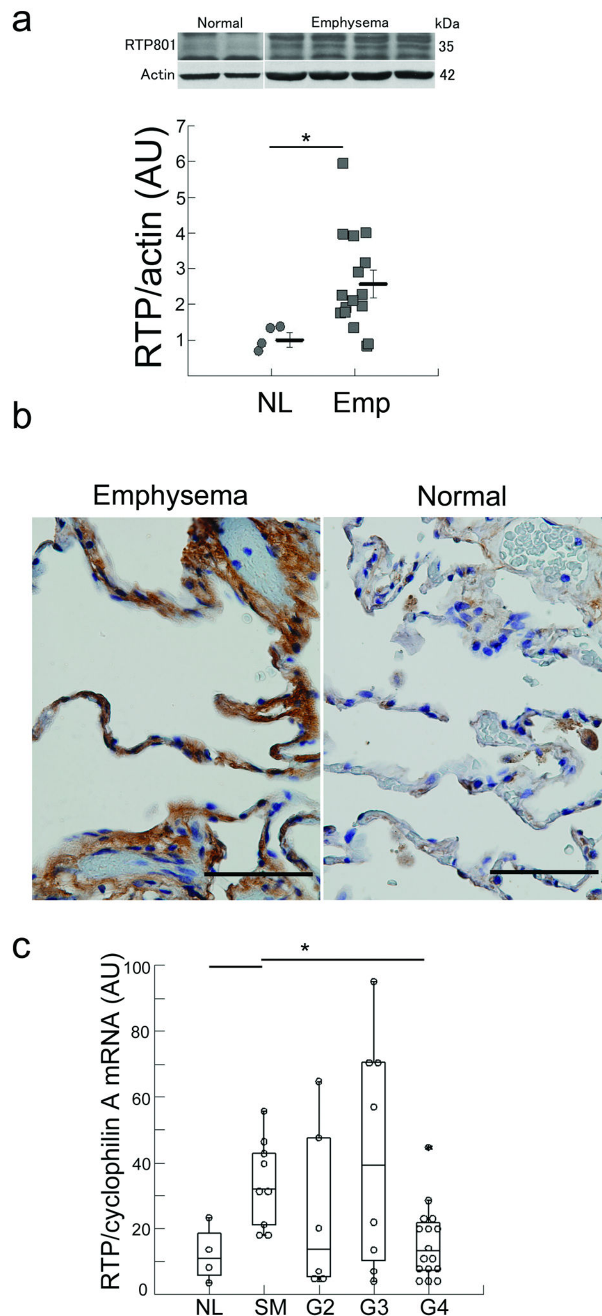

Figure 1. Enhanced expression of *Rtp801* in human emphysematous lungs

(**a**) *Rtp801* expression in normal human lungs (lanes 1 and 2) or emphysematous lungs (GOLD 4) (lanes 3 – 6) (normalized by actin protein expression). (**b**) Histological sections showing increased expression of *Rtp801* (brown) in a lung with emphysema (**left**) when compared with a normal lung (**right**) (arbitrary units (AU); *n* = 4 normal and 16 advanced emphysema lungs). (**c**) Determination of RTP801 mRNA expression in lungs of normal non smokers (n=8), normal smokers (n=13), and smoker patients with Gold stages 2 (n=12), 3

(n=12), and 4 (n=20) (normalized by cyclophilin A; signal intensity in arbitrary units (AU)).*: $P < 0.05$

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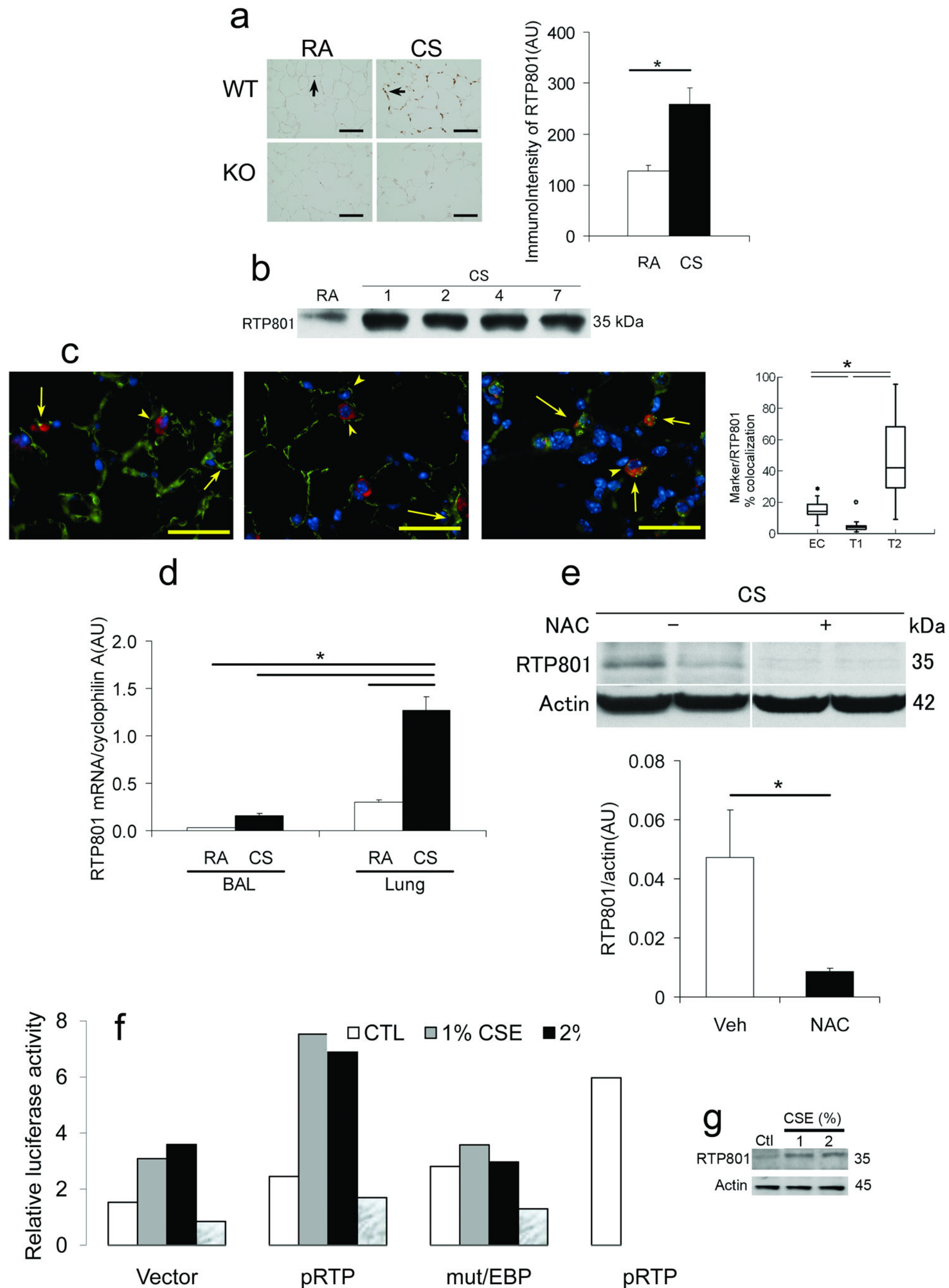

Figure 2. Cigarette smoke – induced upregulation of *Rtp801* expression occurs in lung septal but not resident or infiltrating inflammatory cells and relies on oxidative stress – dependent activation of the CCAAT promoter region

(**a**) Lung *Rtp801* expression (brown, arrows) in wildtype (**upper**) or *Rtp801* $^{-/-}$ (**lower**) mice exposed to RA (**left**) or CSk (**right**) for 7 days (× 50 μm) and expression levels (AU; *n* = 3 and 7, respectively). (**b**) Lung Rtp801 protein expression levels in mice exposed from 0 to 7 days to CSk (pooled *n* = 3 lungs in each time point). (**c**) Lungs costained with Rtp801 (red, arrowheads), nuclei (DAPI, bue), endothelial cells (thombomodulin, **left**), type I epithelial cells (T1α, **middle**), type II cells (ProSpC, **right**) (all in green) in mice exposed to

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CSk for 1 day (superimposed red plus green shown in yellow, arrows). Percent colocalization of alveolar cell specific markers (Marker) (thrombomodulin, T1α, or ProSPC over *Rtp801* expression (10 fields, $n = 3$ lungs/marker; × 50 μm). (**d**) *Rtp801* mRNA expression levels in Bal and lung tissue in wildtype mice exposed to CSk for 1 day or ambient air controls (RA) (AU, $n = 3 – 4$ mice in each group). (**e**) *Rtp801* expression levels in lungs of *Rtp801* wildtype mice treated with NAC (500 mg/kg, i.p.) or vehicle (veh) and exposed to CSk for 1 day (normalized by actin, AU; $n = 4 – 5$ mice in each group). (**f**, **g**) Activity of intact 2.5 kb *Rtp801* promoter or with a point mutation within CCAAT binding site (mut/CEBP) or pGL3 plasmid (Vector) – *Firefly* luciferase in mouse lung fibroblasts (MLF) exposed to media alone (CTL), CSE (1 or 2%), or NAC (10 mM) (positive control: H_2O_2, 250 μM; pRTP+H_2O_2) for 12 h (normalized by *Renilla* luciferase; data representative of 2 independent experiments). (**g**) Expression of Rtp801 in cells from (**f**). *: $P < 0.05$

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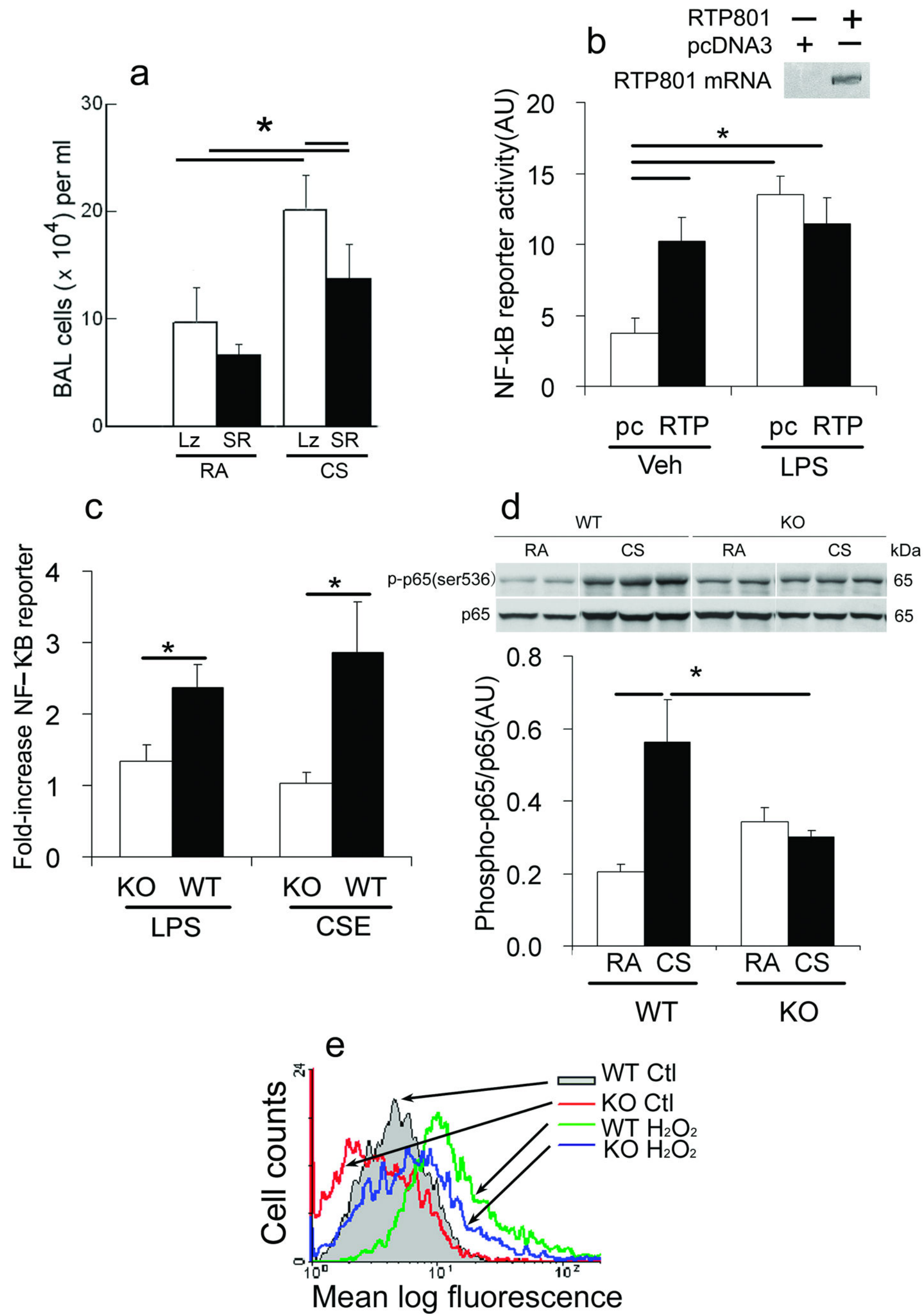

Figure 3. *Rtp801* – dependent NF – κB activation by cigarette smoke

(**a**) Total Bal cells in *Rtp801* wildtype mice intratracheally transduced with AAV5 – expressing IκB super repressor (SR) or β – gal (Lz) for 7 – 10 days in ambient air (RA) or exposed to CSk for 1 day (n = 5 – 6 mice in each group). (**b**) Effect of transfection with *RTP801* – (RTP) or empty vector pcDNA3.1+ (pc) on NF – kB – dependent reporter *Firefly* luciferase activity (lower) in LPS, 1 μg ml^{-1} or vehicle – (veh) treated rat lung endothelial cells (normalized by *Renilla* luciferase activity, AU; n = 3 independent experiments), and *RTP801* mRNA expression in transfected cells (**upper**). (**c**) NF – κB reporter activity in

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wildtype (WT) or *Rtp801* $^{-/-}$ (KO) MLF treated with LPS – (1 ug ml^{-1}) or 1% CSE (normalized AU; $n = 3$ independent experiments). (**d**) Expression levels of phosphorylated Ser536 p65 NF – κB (p – p65 NF – κB) in whole lung lysates of *Rtp801* wildtype and *Rtp801* $^{-/-}$ mice, exposed to CSk for 1 day (normalized by total p65, $n = 3 – 5$ mice in each group). (**e**) Vehicle (Ctl) and H2O2 _ induced (125 μM, 6 h) ROS levels in *Rtp801* wildtype and *Rtp801* $^{-/-}$ MEF. *: $P < 0.05$

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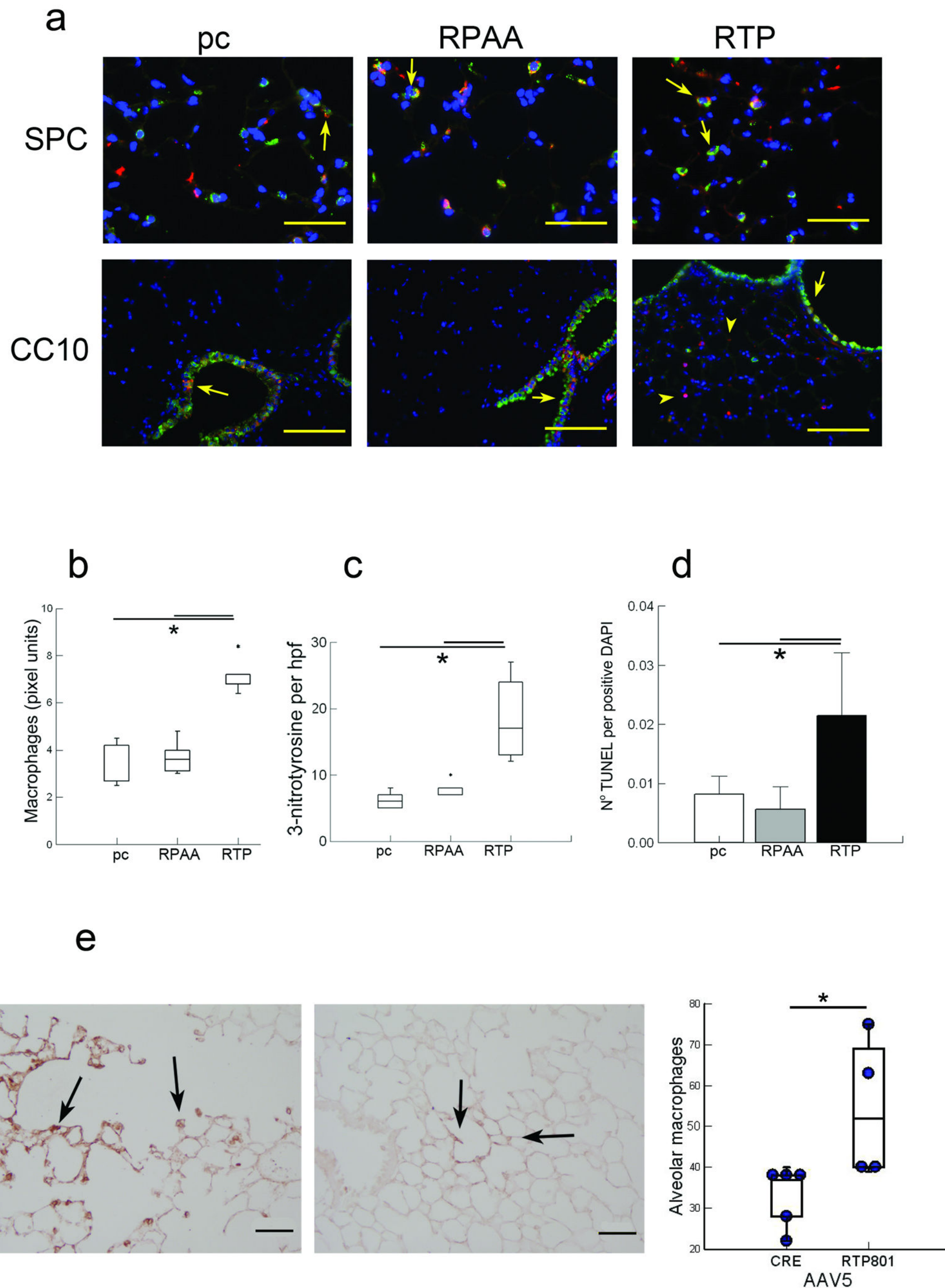

Figure 4. Forced *in vivo* overexpression of human*RTP801* in mouse lungs enhances oxidative stress, inflammation, and alveolar cell apoptosis

Effect of human *RTP801* – expressing (RTP, **right**), mutant *Rtp801 – RPAA* (RPAA, **middle**), or control pcDNA3.1 (pc, **left**) injected i.v. (24 h) in *Rtp801* wildtype lungs costained (**a**) with RTP801 (red), type II cells (ProSPC, red, **upper**), or airway epithelial cells (CC10, green, **lower**) with coexpression highlighted by arrows (in yellow) (upper row: × 50 μm; lower row: × 200 μm). Numbers of infiltrating lung macrophages (**b**), 3 – nitrotyrosine positive cell profiles per high power field (hpf) (**c**), and alveolar cell apoptosis

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(TUNEL, normalized by DAPI – positive nuclei) (**d**) (10 fields, $n = 5 – 10$ mice in each group). (**e**) Infiltrating lung macrophages (brown, **upper**) in alveolar septa along an alveolar duct (arrows) in mice infected i.t. with AAV5 – *RTP801* (**left**) or AAV5 – *Cre* (**right**) (negative control) for 4 weeks with quantification of numbers of alveolar macrophages per high power field (**lower**; 10 fields, $n = 4 – 5$ mice in each group). *: $P < 0.05$.

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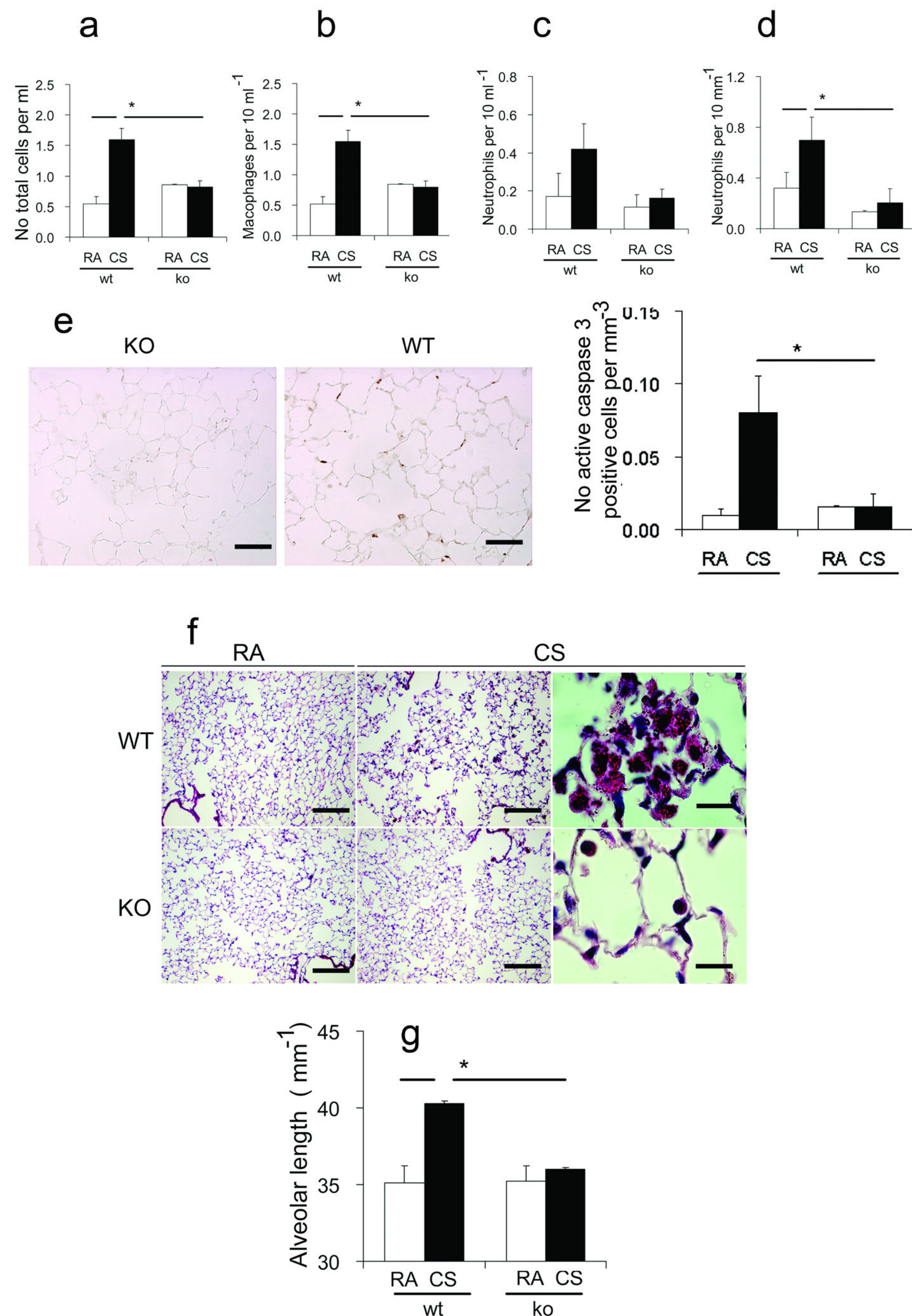

Figure 5. *Rtp801* $^{-/-}$ mice are protected against cigarette smoke – induced pulmonary inflammation, apoptosis, and emphysema

Bal total cell counts (**a**), including numbers of macrophages (**b**), and neutrophils (**c**), infiltrating neutrophils in alveolar lung tissue (**d**) (μm^{-1} alveolar length), active caspase 3 – positive cells (brown, arrows) (**upper**) (× 50 μm) and numbers of active caspase 3 – positive cells (μm^{-1} alveolar septa) (**lower**) in *Rtp801* wildtype and *Rtp801* $^{-/-}$ mice kept in RA or exposed to CSk for 7 days (*n* = 3 and 7 mice, respectively). Alveolar morphology in *Rtp801* wildtype CSk for 6 months (**f**) showing airspace enlargement and large clusters of alveolar

macrophages containing smoking pigment in the cytoplasm when compared with *Rtp801* $^{-/-}$ mouse lungs (× 250 μm, right and middle; × 25 μm, left). Alveolar length represent mean linear intercepts (**g**) (*n* = 5 to 7 mice in each group). *: $P < 0.05$

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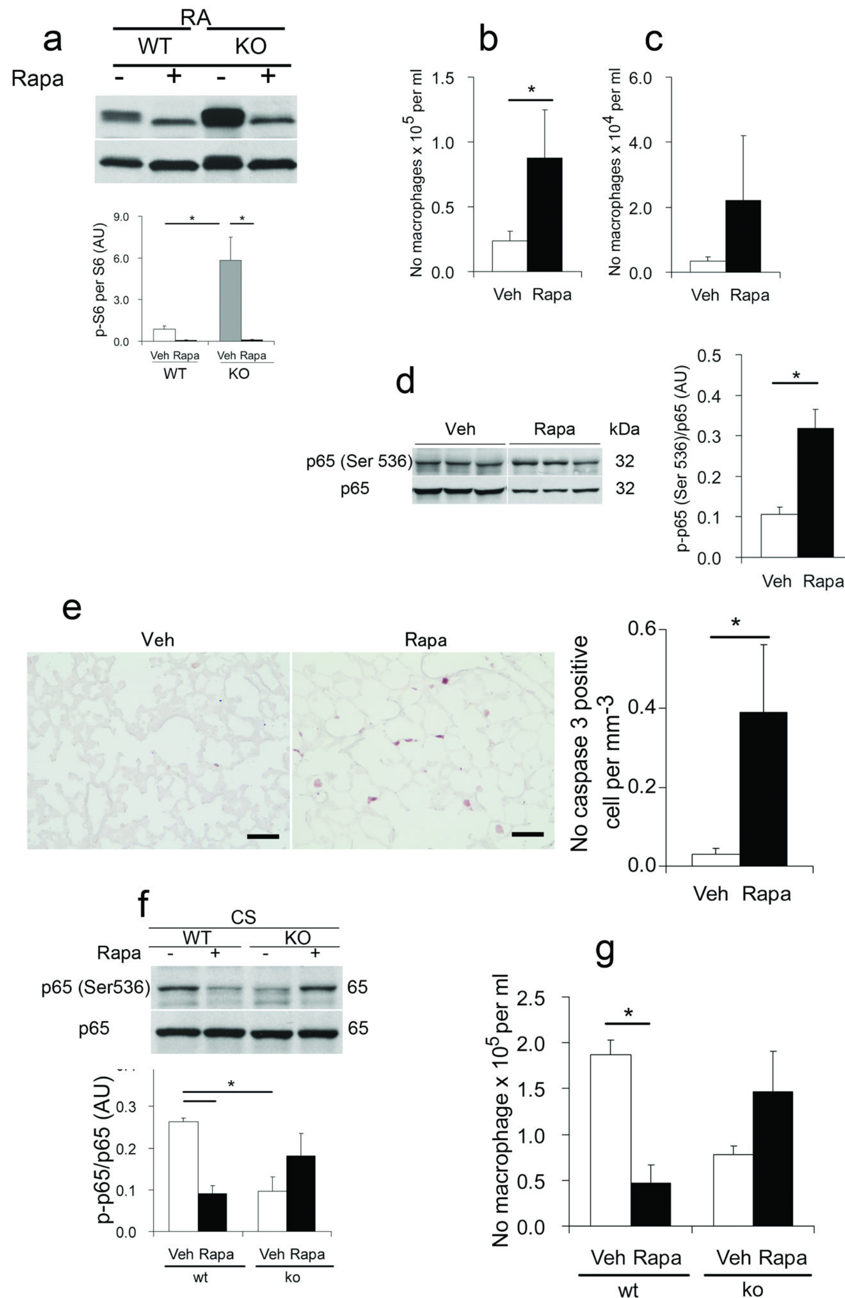

Figure 6. mTOR regulates lung homeostasis and inflammatory responses due to cigarette smoke in *Rtp801* wildtype and *Rtp801* $^{-/-}$ mice

Effect of vehicle (–) or rapamycin (rapa) treatment on phosphorylated serine 235/236 S6 (p – S6) protein expression (normalized by S6, AU) (**a**), on inflammatory cell counts of Bal macrophages (**b**) and neutrophils (**c**), lung p – p65 NF – κB protein expression (**d**) (normalized by p65, AU), on lung expression of active caspase 3 (**e**) (brown, **left**, × 50 μm), and total counts (**right**) of active caspase 3 – positive cellular profiles (normalized by μm alveolar length) in RA – exposed C57Bl6 mice. Effect of vehicle or rapa treatment on p –

p65 NF – kB expression (**f**) (normalized by total p65, AU) and numbers of macrophages (**g**) in Bal fluid from lungs of wildtype and *Rtp801* $^{-/-}$ mice exposed to CSk for 1 day (10 fields, $n = 4/5$ mice in each group). *: $P < 0.05$).

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