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

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Rtp801 (also known as Redd1, and encoded by *Ddit4*), a stress-related protein triggered by adverse environmental conditions, inhibits mammalian target of rapamycin (mTOR) by stabilizing the TSC1-TSC2 inhibitory complex and enhances oxidative stress–dependent cell death. We postulated that Rtp801 acts as a potential amplifying switch in the development of cigarette smoke–induced lung injury, leading to emphysema. Rtp801 mRNA and protein were overexpressed in human emphysematous lungs and in lungs of mice exposed to cigarette smoke. The regulation of Rtp801 expression by cigarette smoke may rely on oxidative stress–dependent activation of the CCAAT response element in its promoter. We also found that Rtp801 was necessary and sufficient for nuclear factor-κB (NF-κB) activation in cultured cells and, when forcefully expressed in mouse lungs, it promoted NF-κB activation, alveolar inflammation, oxidative stress and apoptosis of alveolar septal cells. In contrast, Rtp801 knockout mice were markedly protected against acute cigarette smoke–induced lung injury, partly via increased mTOR signaling, and, when exposed chronically to cigarette smoke, against emphysema. Our data support the notion that Rtp801 may represent a major molecular sensor and mediator of cigarette smoke–induced lung injury.

Emphysematous lung destruction is a major component of chronic obstructive pulmonary disease (COPD), a highly prevalent disease predominantly caused by exposure to cigarette smoke and environmental pollutants[1]. Cigarette smoke–triggered oxidative stress initiates lung inflammation and excessive extracellular matrix proteolysis 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 (ref. 8) (or Redd1 for regulated in development and DNA damage responses[9]) was identified on the basis of its induction by hypoxia[8] or DNA damage[9]. Brain ischemia leads to upregulated neuronal Rtp801 expression[8], and induction of Rtp801 protein in the retinal inner nuclear cell layer is required for neural retina and central vessel endothelial cell death in mice with oxygen-induced retinopathy[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 mTOR[11,12] via activation of TSC2 (tuberin), a negative regulator of mTOR[11,13,14]. Decreased mTOR activity downregulates hypoxia inducible factor-1 (HIF-1)-dependent vascular endothelial growth factor (VEGF), which has been linked to experimental and human emphysema[1,15,16].

Here we addressed whether the Rtp801-mTOR axis might integrate environmental stresses due to cigarette smoke with the activation of inflammation and cell death signals, leading to lung tissue damage and emphysematous destruction.

RESULTS

Increased Rtp801 expression in lung pathology

We found significantly upregulated RTP801 protein expression ($P < 0.05$) in lungs of individuals 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 individuals with mild to moderate COPD had increased expression of RTP801 mRNA, whereas lungs

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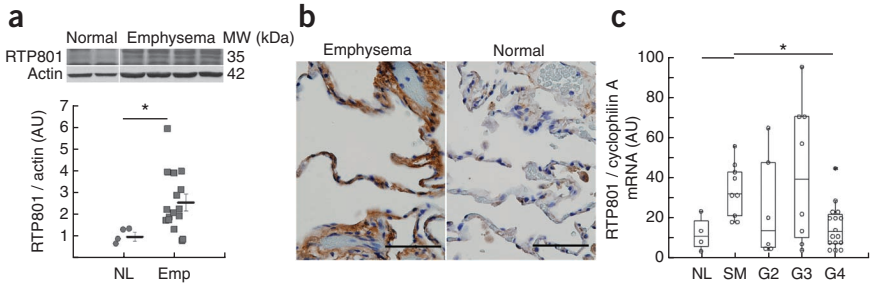


Figure 1 Enhanced expression of RTP801 in human emphysematous lungs. (**a**) RTP801 protein expression in normal human lungs (NL; $n = 4$) or advanced emphysematous (Emph; $n = 16$) lungs (normalized to actin protein expression, arbitrary units (AU)). (**b**) Histological sections showing increased expression of RTP801 (brown) in a lung with emphysema (left) compared to a normal lung (right). Scale bar, 100 μm. (**c**) Determination of RTP801 mRNA expression in lungs of normal nonsmokers (NL, $n = 8$), normal smokers (SM, $n = 13$), and smokers with COPD severity grading of 2 (mild, $n = 12$), 3 (moderate, $n = 12$), and 4 (severe, $n = 20$) according to the scoring of Global Initiative for Chronic Obstructive Lung Disease (G). All values are normalized to cyclophilin A. $^*P < 0.05$. Data are represented as means ± s.e.m.

of individuals with severe disease expressed similar levels of RTP801 transcript as compared to the lungs of normal nonsmokers (**Fig. 1c** and **Supplementary Table 1**). These findings suggest that RTP801 may undergo post-transcriptional 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 cigarette smoke–induced lung oxidative stress[18]. Mice exposed to cigarette smoke for up to 7 d showed increased lung expression of Rtp801protein in alveolar septa by immunohistochemistry (**Fig. 2a**) and western blot analyses (**Fig. 2b**). Alveolar type II pneumocytes showed the highest amounts of Rtp801, followed by endothelial cells and type I pneumocytes, which had minimal expression (**Fig. 2c**). Of note, expression of Rtp801 mRNA and protein seemed to predominate in alveolar septal cells rather than inflammatory cells, given the more modest expression of Rtp801 mRNA (**Fig. 2d**) and protein abundance in cells obtained by bronchoalveolar lavage (composed predominantly by inflammatory cells) and the lack of the more sensitive immunohistochemistry signal in macrophages (data not shown). Mice exposed to cigarette smoke for 4–6 months also showed high Rtp801 mRNA expression (**Supplementary Fig. 1a**)[19,20].

Role of oxidative stress in induction of Rtp801 expression

Consistent with previous observations that oxidative stress induces Rtp801 expression *in vitro*[8,21], the antioxidant *N*–acetyl–L–cysteine (NAC, 10 mM) completely prevented lung Rtp801 protein upregulation caused by cigarette smoke exposure for 1 d (**Fig. 2e**). Consistent with these results, lungs of *Nfe2l2*$^{-/-}$ mice, which have heightened sensitivity to cigarette smoke–induced lung injury due to lack of the master antioxidant transcription factor Nrf2 (ref. 19), showed increased Rtp801 mRNA signal when compared with wild-type littermates chronically exposed to cigarette smoke (**Supplementary Fig. 1a**). However, the resistant mouse strain CD-1 and the more sensitive strain C57BL6/J had similar levels of Rtp801 mRNA when exposed to cigarette smoke for 1 d (data not shown).

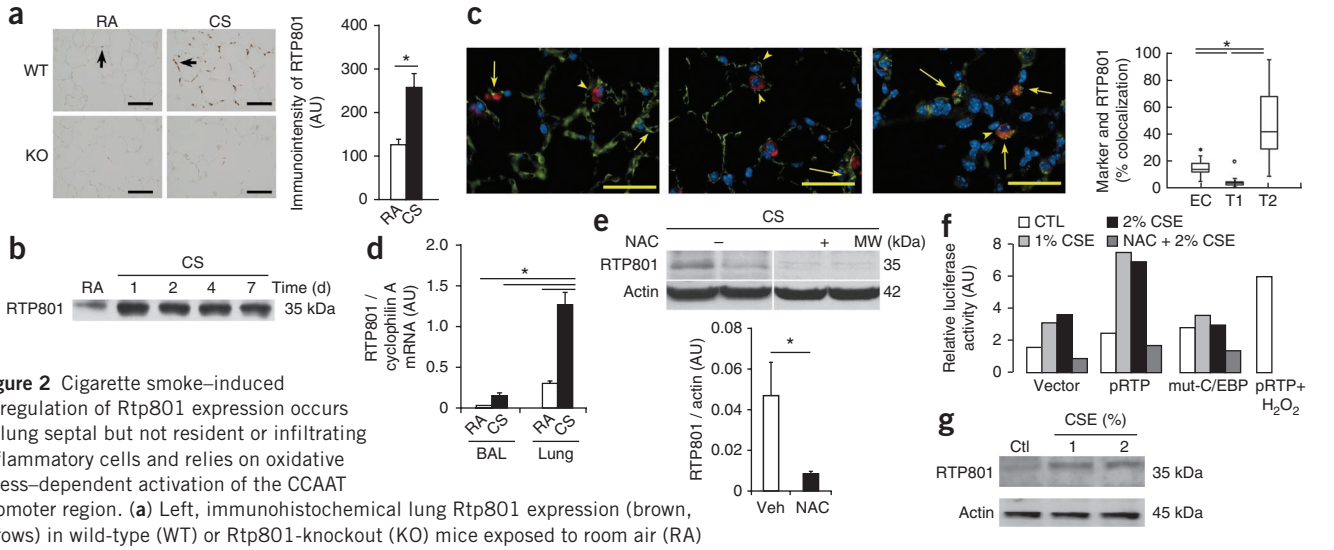


Figure 2 Cigarette smoke–induced upregulation of Rtp801 expression occurs in lung septal but not resident or infiltrating inflammatory cells and relies on oxidative stress–dependent activation of the CCAAT promoter region. (**a**) Left, immunohistochemical lung Rtp801 expression (brown, arrows) in wild-type (WT) or Rtp801-knockout (KO) mice exposed to room air (RA) or cigarette smoke (CS) for 7 d. Right, quantification of the staining intensity ($n = 3$ for room air and for $n = 7$ cigarette smoke treatment groups). Scale bar, 50 μm. (**b**) Lung Rtp801 protein expression in mice exposed from day 0 to day 7 to cigarette smoke (pooled $n = 3$ lungs in each time point). (**c**) Left, lungs costained for Rtp801 (red, arrowheads), nuclei (DAPI, blue), endothelial cells (thombomodulin, left), type I epithelial cells (the differentiation protein T1α or podoplanin, middle), type II cells (prosurfactant C (ProSpC)), right) (all in green) in mice exposed to cigarette smoke for 1 d (superimposed red plus green shown in yellow, arrows). Right, percentage colocalization of alveolar cell–specific markers (Marker; thrombomodulin for endothelial cells (EC), T1α for type I epithelial cells (T1) or ProSpC for type II cells (T2)) over Rtp801 expression (ten fields, $n = 3$ lungs per marker). Scale bars, 50 μm. (**d**) Rtp801 mRNA expression levels in bronchoalveolar lavage and lung tissue in wild-type mice exposed to cigarette smoke for 1 d or room air ($n = 3$ or 4 mice in each group). (**e**) Rtp801 expression in lungs of wild-type mice treated with NAC (500 mg per kg body weight, intraperitoneally) or vehicle (veh) and exposed to cigarette smoke for 1 d (normalized to actin; $n = 4$ or 5 mice in each group). (**f**) Activity of the intact 2.5-kb *DDIT4* promoter (pRTP) or of the promoter with a loss-of-function mutation within the CCAAT binding site (mut-C/EBP) or pGL3 plasmid (Vector)–firefly luciferase in MLFs exposed to medium alone (CTL), CSE (1% or 2%), or NAC + 2% CSE (10 mM) (positive control: H_2O_2, 250 μM; pRTP + H_2O_2) for 12 h (normalized by *Renilla* luciferase; data representative of two independent experiments). (**g**) Expression of Rtp801 protein in cells from **f**. $^*P < 0.05$. Data are represented as means ± s.e.m.

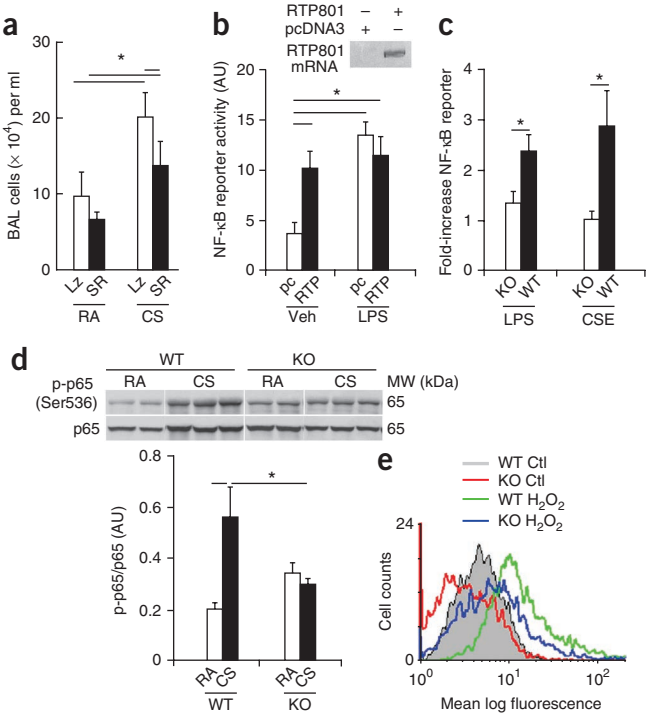


Figure 3 Rtp801-dependent NF-κB activation by cigarette smoke. (**a**) Total bronchoalveolar lavage (BAL) cells in wild-type mice intratracheally transduced with AAV5–expressing IκB superrepressor (SR) or β-galactosidase (Lz) for 7–10 d in room air or exposed to cigarette smoke for 1 d ($n = 5$ or 6 mice in each group). (**b**) Effect of transfection with *DDIT4* (RTP) or empty vector pcDNA3.1+ (pc) on NF-κB–dependent reporter *Firefly* luciferase activity (bottom) in LPS-treated (1 μg ml^{-1}) or vehicle-treated rat lung endothelial cells (normalized to *Renilla* luciferase activity; $n = 3$ independent experiments), and RTP801 mRNA expression in transfected cells (top). (**c**) NF-κB reporter activity in wild-type or Rtp801-knockout MLFs treated with LPS (1 μg per ml) or 1% CSE (normalized to PBS-treated cells, $n = 3$ independent experiments). (**d**) Expression and densitometric quantification of phosphorylated Ser536 of p65 NF-κB (p–p65) in whole-lung lysates of wild-type and Rtp801-knockout mice, exposed to cigarette smoke for 1 d (expression normalized to total p65 (AU), $n = 3$–5 mice in each group). *$P < 0.05$. (**e**) Vehicle (Ctl) and H_2O_2-induced (125 μM, 6 h) ROS levels measured by intracellular 2′, 7′-dichlorofluorescein diacetate fluorescence by FACS in MEFs from wild-type and Rtp801-knockout lungs (*x* axis: mean log fluorescence (AU); *y* axis: cell counts). Data are represented as means ± s.e.m.

The *DDIT4* promoter contains regulatory elements that bind the transcription factors ELK1, CCAAT/enhancer-binding protein (C/EBP), hepatic nuclear factor-4, NF-κB, p53 and HIF-1α[21]. The cigarette smoke induction of reporter plasmids driven by the 2.5-kb *DDIT4* promoter[21] involved the activation of the CCAAT motif, as the mutation of AA to CC in the sequence GATGAAACAC of its response element reduced cigarette smoke–dependent luciferase activity to baseline levels in mouse lung fibroblasts (MLFs) (**Fig. 2f**), the human bronchial epithelial cell line Beas2B (**Supplementary Fig. 1b**) and the human fetal kidney epithelial cell line HEK293 (data not shown). Enhanced expression of endogenous Rtp801 protein in duplicate cell samples paralleled the induction of the *Ddit4* promoter–driven reporter plasmid (**Fig. 2g** and **Supplementary Fig. 1c**). NAC added with cigarette smoke extract (CSE) prevented the activation of the *DDIT4* promoter-bearing reporter construct in MLFs (**Fig. 2f**).

RTP801 overexpression and activation of NF-κB

Cigarette smoke–associated oxidative stress[22] may promote lung inflammation through NF-κB signaling[23]. Accordingly, inhibition of NF-κB activation in lungs infected with adenovirus inhibitor of κBα (IκBα) superrepressor[24] significantly ($P < 0.05$) decreased lipopolysaccharide (LPS, positive control)-induced and, notably, cigarette smoke–induced accumulation of inflammatory cells in the bronchoalveolar lavage (**Fig. 3a** and **Supplementary Fig. 2a–c**). However, expression of the IκBα superrepressor had no effect on cigarette smoke–dependent induction of the oxidative stress markers 3-nitrotyrosine (**Supplementary Fig. 2d**) and 8-oxo-2-deoxyguanosine (data not shown) as compared with cigarette smoke–exposed control mice. The upregulation of Rtp801 mRNA levels driven by acute cigarette smoke exposure was not affected by expression of the IκB superrepressor (**Supplementary Fig. 2e**). LPS, an activator of NF-κB, did not alter Rtp801 mRNA expression in challenged lungs when compared with PBS-instilled lungs, and, conversely, the IκB superrepressor did not affect lung Rtp801 mRNA levels after treatment with LPS (**Supplementary Fig. 2f**) or in cultured A549 cells treated with CSE or tumor necrosis factor-α (**Supplementary Fig. 2g,h**).

Human *DDIT4* 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, as Rtp801-knockout MLFs did not activate luciferase expression from the NF-κB–driven reporter plasmid when stimulated with CSE or LPS (**Fig. 3c**).

In ambient room air conditions, Rtp801-knockout lungs showed a trend toward enhanced baseline phosphorylation of Ser536 of NF-κB p65 (which closely correlates with NF-κB transcriptional activation[25]). However, after exposure to cigarette smoke, only wild-type mice showed a sizable NF-κB response with phosphorylation of p65 on Ser536, whereas this response was abolished in Rtp801-knockout mice (**Fig. 3d**); MLFs showed similar CSE-induced NF-κB responses (**Supplementary Fig. 3a**). We also detected higher mRNA expression of the gene encoding NF-κB–dependent chemokine macrophage inflammatory protein-2 (Cxcl2)[26] in lungs from wild-type mice exposed to cigarette smoke, compared with similarly treated Rtp801-knockout mice (**Supplementary Fig. 3b**). The stress– and cigarette smoke–inducible p38 mitogen-activated protein kinase was probably not involved in the NF-κB activation induced by cigarette smoke, as the p38 mitogen-activated protein kinase inhibitor SB239063 did not lessen phosphorylation of Ser536 or Ser276 of NF-κB p65 in mice exposed to cigarette smoke (**Supplementary Fig. 3c–e**).

Further supporting the observed link between oxidative stress and NF-κB activation[26], Rtp801-knockout mouse embryonic fibroblasts (MEFs) contained lower amounts of reactive oxygen species (ROS) than wild-type cells, either at baseline or after treatment with H_2O_2 from 6 h to 24 h (**Fig. 3e** and **Supplementary Fig. 3f**). Likewise, the mouse lung epithelial cell line MLE-15 (ref. 27) showed less oxidative stress caused by H_2O_2 after it was transduced with a specific siRNA that lowered Rtp801 mRNA levels by approximately 50%, as compared with Rtp801-replete cells given a scrambled siRNA (**Supplementary Fig. 3g**). These findings are in line with higher mRNA expression of the gene encoding the inducible antioxidant heme oxygenase-1 in cigarette smoke–exposed Rtp801-knockout lungs compared with wild-type controls **Supplementary Fig. 3**). Together, these data position the stress-dependent upregulation of Rtp801 by cigarette smoke upstream of NF-κB activation both *in vivo* and *in vitro*.

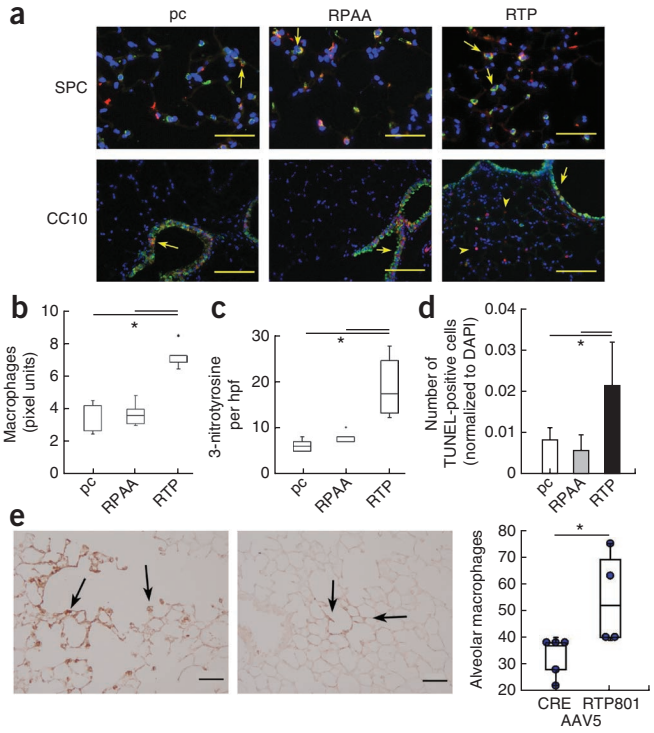
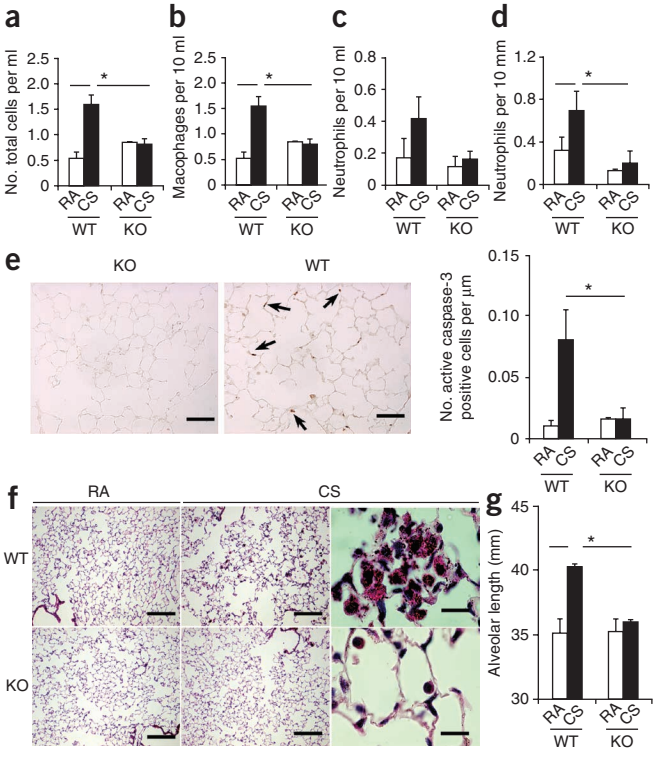


Figure 4 Forced *in vivo* overexpression of human RTP801 in mouse lungs enhances oxidative stress, inflammation and alveolar cell apoptosis. (**a**) Lung expression of Rtp801 after *in vivo* lung transduction of backbone vector pcDNA3.1 (pc), mutant *DDIT4*-RPAA (RPAA) or *DDIT4* (RTP) cDNA vectors injected intravenously (24 h before harvesting) in wild-type mice. Coimmunofluorescence of Rtp801 (red), type II cells (ProSpC (SPC), red) or airway epithelial cells (with clara cell antigen 10 (CC10), green), with coexpression highlighted by arrows (in yellow). Scale bars: top row, 50 μm; bottom row: 200 μm. (**b**–**d**) Numbers of infiltrating lung macrophages (**b**), 3-nitrotyrosine–positive cell profiles per high-power field (hpf) (**c**) and alveolar cell apoptosis (TUNEL, normalized by DAPI-positive nuclei) (**d**) (ten fields, $n = 5$–10 mice in each group). (**e**) Infiltrating lung macrophages (brown) in alveolar septa along an alveolar duct (arrows) in mice infected intratracheally with AAV5-*DDIT4* (left) or AAV5-*Cre* (negative control, right) for 4 weeks, with quantification to the far right of the numbers of alveolar macrophages per high-power field (ten fields, $n = 4$ or 5 mice in each group). Scale bars, 100 μm. $^*P < 0.05$. Data are represented as means ± s.e.m.

RTP801 overexpression in mouse lungs causes alveolar injury

We next addressed whether forced overexpression of human RTP801 reproduces the mouse lung phenotypes caused by cigarette smoke exposure. Intratracheally instilled human *DDIT4* cDNA led to increased Rtp801 protein expression, localized predominantly in alveolar type II cells rather than small airway cells (assessed by an antibody that recognizes both the more abundant mouse Rtp801 and also the human protein) (**Fig. 4a**). In contrast, lungs instilled with *DDIT4*-RPAA, in which the conserved arginine and proline in the 14-3-3 binding domain are mutated to alanine, rendering the encoded protein unable to inhibit TORC1 activity[28], or with the empty vector pcDNA3 had substantially lower expression of Rtp801 when compared with *DDIT* cDNA. Overexpression of RTP801 triggered enhanced lung inflammation, oxidative stress and alveolar cell death when compared with *DDIT4*-RPAA or pcDNA3 (**Fig. 4b**–**d** and **Supplementary Fig. 4a**).



Intratracheal infection of wild-type mice with adeno-associated virus serotype 5 (AAV5) containing *DDIT4* (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 AAV5-*Cre*–transduced lungs (**Fig. 4e**). However, we did not observe significant ($P > 0.05$) airspace enlargement, such is often seen with longer (such as 6 months) exposures to cigarette smoke (data not shown).

Rtp801 mediates cigarette smoke induced pulmonary injury

Rtp801-knockout mice have normal lung structure at 1, 2, 4 and 12 weeks of age when compared with wild-type littermates, on the basis of analyses of lung sections stained with H&E, elastin, type II cell prosurfactant protein C expression and terminal airway epithelial Clara cell antigen immunohistochemistry (data not shown). Having found that forced RTP801 expression in mouse lungs results in oxidative stress, apoptosis and inflammation, we next assessed whether Rtp801 expression is necessary for cigarette smoke–induced lung pathology. Rtp801-knockout

Figure 5 Rtp801-knockout mice are protected against cigarette smoke–induced pulmonary inflammation, apoptosis and emphysema. (**a**–**d**) Bronchoalveolar lavage total cell counts (**a**), including numbers of macrophages (**b**) and neutrophils (**c**), and infiltrating neutrophils in alveolar lung tissue (per μm alveolar length) (**d**). (**e**) Left, active caspase-3–positive cells from mice exposed to cigarette smoke for 7 d (brown, arrows). Right, numbers of active caspase-3–positive cells (per μm alveolar septa) (right) in wild-type and Rtp801-knockout mice kept in room air or exposed to cigarette smoke (CS) for 7 d ($n = 3$ and 7 mice, respectively). Scale bar, 50 μm. (**f**) Alveolar morphology in wild-type mice exposed to cigarette smoke for 6 months versus room air, showing airspace enlargement (left and middle) and large clusters of alveolar macrophages containing smoking pigment (right) in the cytoplasm and compared with Rtp801-knockout mouse lungs under the same exposures. Scale bars, left and middle images, 250 μm; right images, 25 μm. (**g**) Alveolar sizes of mice in **f** assessed by alveolar length determined by mean linear intercepts ($n = 5$ to 7 mice in each group). $^*P < 0.05$. Data are represented as means ± s.e.m.

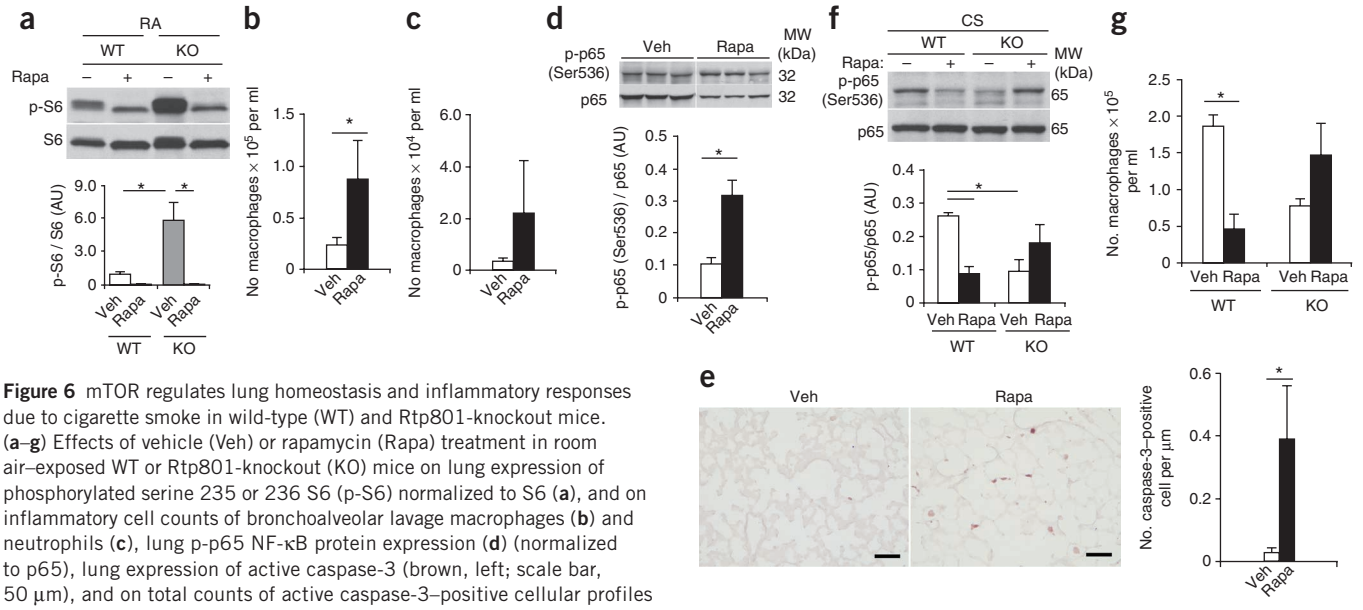


Figure 6 mTOR regulates lung homeostasis and inflammatory responses due to cigarette smoke in wild-type (WT) and Rtp801-knockout mice. (**a**–**g**) Effects of vehicle (Veh) or rapamycin (Rapa) treatment in room air–exposed WT or Rtp801-knockout (KO) mice on lung expression of phosphorylated serine 235 or 236 S6 (p-S6) normalized to S6 (**a**), and on inflammatory cell counts of bronchoalveolar lavage macrophages (**b**) and neutrophils (**c**), lung p-p65 NF-κB protein expression (**d**) (normalized to p65), lung expression of active caspase-3 (brown, left; scale bar, 50 µm), and on total counts of active caspase-3–positive cellular profiles (normalized to µm alveolar length) (right) in room air–exposed wild-type mice (**e**). (**f**,**g**) Effect of vehicle (Veh) or rapamycin (Rapa) treatment on expression of phosphorylation of Ser536 of p65 NF-κB (p-p65) normalized to total p65 (**f**) and numbers of macrophages (**g**) in bronchoalveolar lavage fluid from lungs of wild-type (WT) and Rtp801-knockout (KO) mice exposed to cigarette smoke for 1 d (ten fields, $n = 4$ or 5 mice in each group). $^*P < 0.05$. Data are represented as means ± s.e.m.

mice, when exposed to cigarette smoke for up to 7 d, showed complete protection against acute inflammation, including reduced numbers of total cells (**Fig. 5a**), macrophages (**Fig. 5b**) and neutrophils (**Fig. 5c**) in bronchoalveolar lavage fluid and reduced neutrophil influx in lung parenchyma (**Fig. 5d**) when compared with similarly treated wild-type mice. Rtp801-knockout mice also showed significantly lower ($P < 0.05$) numbers of apoptotic cells, as assessed by immunohistochemistry staining of activated caspase-3 (**Fig. 5e**) and poly-ADP ribose polymerase cleavage in immunoblots (**Supplementary Fig. 4b**). Furthermore, when exposed to cigarette smoke, Rtp801-knockout mice had increased expression of HIF-1α–dependent lung protective genes, such as *Vegfa* (coding for the 120-kDa Vegfa variant), and *Slc2a1*, the gene encoding glucose transporter-1 (**Supplementary Fig. 3i**,**j**).

Consistent with their protection against pathology caused by short-term cigarette smoke exposure, Rtp801-knockout mice had preserved alveolar structure after a 6-month exposure to cigarette smoke, according to a previously published procedure[30], with no increases in mean linear intercepts (versus room air (ambient air)-exposed mice) and sparse accumulation of intra-alveolar macrophages. In contrast, wild-type mice showed the classic morphologic features[30] of airspace enlargement and accumulation of alveolar macrophages filled with smoking pigment (a tannish-brown pigment consisting of small, needle-like lysosomal deposits of aluminum silicate contained in cigarette smoke) (**Fig. 5f**,**g**). These findings were accompanied by decreased expression and nuclear accumulation of NF-κB in Rtp801-knockout lungs when compared with wild-type lungs (**Supplementary Fig. 4c**). These results indicate that Rtp801 might have a crucial role in the pathogenesis of experimental cigarette smoke–induced pulmonary injury and emphysema.

Rtp801 regulation of mTOR and lung injury

Rtp801 negatively regulates mTOR activity by disassociating the inhibitory protein 14-3-3 from Tsc2, and allowing the Tsc1-Tsc2 complex then to block mTOR activity[11,28], as evidenced by decreased phosphorylation of S6 kinase and its downstream targets S6 and 4E-BP1[31]. Accordingly, we found enhanced S6 phosphorylation on Ser235 or Ser236 (p-S6) in Rtp801-knockout, room air–exposed lungs and MLFs compared with wild-type lungs and MLFs, respectively (**Fig. 6a** and **Supplementary Fig. 5a**,**b**). The selective mTORC1 inhibitor rapamycin pronouncedly blocked p-S6 in mouse lungs and MLFs under control conditions (**Fig. 6a** and **Supplementary Fig. 5b**).

To further define the potential function of mTOR (specifically, mTORC1) in lung homeostasis in response to room air or cigarette smoke, we investigated the influence of rapamycin on pulmonary inflammation and apoptosis in wild-type mice under both conditions. Cigarette smoke led to a marked increase in p-S6 expression in total wild-type lung homogenates (which represent both alveolar septal and infiltrating inflammatory cells) on day 1 when compared with room air (**Supplementary Fig. 5a**). In room air–kept wild-type mice, rapamycin led to increased inflammatory cells in the bronchoalveolar lavage fluid (**Fig. 6b**,**c**), with lung-enhanced expression of phosphorylated Ser536 on NF-κB p65 (**Fig. 6d**) and heightened numbers of apoptotic alveolar cells in lung parenchyma (**Fig. 6e**). Furthermore, rapamycin pretreatment partially abrogated the protection against cigarette smoke–induced inflammation observed in the Rtp801-knockout mice (**Fig. 6f**,**g**). Paradoxically, rapamycin decreased alveolar inflammation and phosphorylated Ser536 of NF-κB p65 in wild-type mice exposed to cigarette smoke (**Fig. 6f**,**g**). Together, these findings indicate that mTOR activity contributes to maintenance of lung homeostasis under normal conditions and partly accounts for the resistance to cigarette smoke–induced alveolar inflammation in Rtp801-knockout mice.

DISCUSSION

Our findings indicate that Rtp801 has a crucial role in the pathogenesis of alveolar injury caused by cigarette smoke, amplifying inflammatory and cell death responses by negatively regulating mTOR signaling and activating NF-κB. Enhanced NF-κB activation *in vitro* and particularly *in vivo* leads to expression of cytokines (for example, macrophage inflammatory protein-2α (ref. 32)) responsible for the recruitment of

neutrophils and macrophages[33]. Moreover, Rtp801 also directs alveolar cell apoptosis due to acute cigarette smoke, which might further promote lung inflammation, oxidative stress and extracellular matrix degradation[3]. In rodents, acute cigarette smoke exposure causes fragmentation of lung elastin[34], generating chemotactic elastin peptides[5] and a collagen degradation product, the tripeptide proline-glycine-proline, which binds CXCR-2 and stimulates neutrophil lung infiltration and alveolar destruction[7]. Alveolar injury caused by acute cigarette smoke exposure might therefore have relevance to chronic emphysematous lung destruction. Our finding that alveolar type II and endothelial cells (rather than inflammatory cells) upregulate Rtp801 in response to cigarette smoke underscores the key role of these cell types in the pathogenesis of alveolar destruction in emphysema and the 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 cigarette smoke 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 as a determinant of tissue injury caused by cigarette smoke. Indeed, we found that RTP801 overexpression in lung epithelial cells *in vivo* not only promotes septal cell apoptosis[8] but also enhances lung macrophage infiltration. These properties may promote aseptic innate inflammation[39] and therefore contribute to the persistence of inflammation in COPD despite smoking cessation, via stimulation of autoimmunity[40,41]. Moreover, it seems that Rtp801 does not directly affect the antioxidant master transcription factor Nrf-2 signaling, on the basis of gene expression profiling of wild-type versus Rtp801-knockout lungs exposed to cigarette smoke (T.Y., E.F., T.R., S.B. & R.M.T., unpublished data). 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 cigarette smoke and LPS (no general abnormalities were seen in peripheral blood cells in Rtp801-knockout mice (E.F., unpublished observation)), allowing for further amplification of oxidant generation by inflammatory and parenchymal cells[42]. The finding that a mutant RTP801, which does not activate Tsc-2, did not increase lung injury when acutely overexpressed potentially implies that inhibition of mTOR signaling by Rtp801 may be mechanistically involved in lung inflammation and alveolar cell apoptosis, which is characteristic of cigarette smoke–induced lung injury[43]. Rtp801 may therefore link environmental stresses, and the ensuing oxidative stress, with the activation of innate immunity via down-regulation of mTOR signaling.

Consistent with the inhibitory role of Rtp801 in mTOR signaling, the cigarette smoke–resistant Rtp801-knockout mice had more lung mTOR signaling under normal conditions when compared with susceptible wild-type mice. Of note, we also found that, when compared with wild-type mouse lungs, Rtp801-knockout mouse lungs express higher amounts of lung-protective proteins, including $VEGF_{120}$ and the inducible heme oxygenase-1, which participates in catalytic reactions leading to synthesis of antioxidant and anti-inflammatory metabolites such as carbon monoxide, bilirubin and ferrous iron[44,45]. The protective role of enhanced mTOR signaling in Rtp801-knockout lungs against acute cigarette smoke was disrupted by rapamycin, leading to alveolar inflammation and NF-κB phosphorylation in cigarette smoke–exposed mice. These results and the observed alveolar injury in wild-type mice by rapamycin in room air are in line with the described actions of rapamycin in promoting inflammation or apoptosis[46–48]. However, mTOR is a key 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 (that is, in alveolar compared to inflammatory cells) regulation of RtP801 may explain the finding of increased p-S6 expression in wild-type lungs exposed to cigarette smoke. In contrast to the effects in Rtp801-knockout mice, rapamycin protected against acute cigarette smoke–induced inflammation in wild-type mice. This beneficial effect of rapamycin may be due to blockade of mTOR activation in inflammatory cells by cigarette smoke–mediated Akt protein kinase phosphorylation (ref. 49 and T.Y. and R.M.T., unpublished observations) or inhibitor of κB kinase-β activation[50]. These opposing roles of mTOR are supported by the observations that rapamycin causes lymphocytic pneumonitis in transplant recipients[51], whereas it protects against experimental asthma[52]. The timing and lung cell targets of mTOR inhibition might therefore be essential to define its beneficial and pathological roles in disease (**Supplementary Fig. 6**).

In conclusion, our data provide new insight into the role of Rtp801 in stress-response sensing as an integrator of cigarette smoke–induced oxidative stress, NF-κB activation and alveolar cell apoptosis, which are all ultimately involved in the pathogenesis of emphysema. Genes such as Rtp801 that are involved in environmental stress responses, particularly those converging on mTOR, may provide first-tier tissue responses before the action of inflammation, extracellular matrix proteolysis or triggers of apoptosis.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

T.Y., E.F. and R.M.T. designed the experiments, analyzed the data and composed the manuscript; T.Y., I.M., A.K.B., J.B., M.P., L. Zhang, A. Gandjeva, L. Zhen, U.C., T.M., A.R., E.B., H.A., N.N., A. Gelfand and I.P. performed *in vitro* and *in vivo* experiments; R.K.T., T.R., T.S. and S.B. provided mouse lung samples for Rtp801 expression studies; G.C. provided normal human lung samples for Rtp801 expression studies; M.M. and S.D.S. performed the chronic exposure to cigarette smoke.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Yoshida, T. & Tuder, R.M. Pathobiology of cigarette smoke–induced chronic obstructive pulmonary disease. *Physiol. Rev.* **87**, 1047–1082 (2007).
2. Tuder, R.M., Yoshida, T., Arap, W., Pasqualini, R. & Petrache, I. Cellular and molecular mechanisms of alveolar destruction in emphysema: an evolutionary perspective. *Proc. Am. Thorac. Soc.* **3**, 503–510 (2006).
3. Tuder, R.M. *et al.* Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade. *Am. J. Respir. Cell Mol. Biol.* **29**, 88–97 (2003).
4. Ryter, S.W., Chen, Z.H., Kim, H.P. & Choi, A.M. Autophagy in chronic obstructive pulmonary disease: homeostatic or pathogenic mechanism? *Autophagy* **5**, 235–237 (2009).
5. Houghton, A.M. *et al.* Elastin fragments drive disease progression in a murine model of emphysema. *J. Clin. Invest.* **116**, 753–759 (2006).
6. Petrache, I. *et al.* Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat. Med.* **11**, 491–498 (2005).
7. Weathington, N.M. *et al.* A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat. Med.* **12**, 317–323 (2006).
8. Shoshani, T. *et al.* Identification of a novel hypoxia-inducible factor 1–responsive gene, RTP801, involved in apoptosis. *Mol. Cell. Biol.* **22**, 2283–2293 (2002).
9. Ellisen, L.W. *et al.* REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. *Mol. Cell* **10**, 995–1005 (2002).
10. Brafman, A. *et al.* Inhibition of oxygen-induced retinopathy in RTP801-deficient mice. *Invest. Ophthalmol. Vis. Sci.* **45**, 3796–3805 (2004).
11. Brugarolas, J. *et al.* Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev.* **18**, 2893–2904 (2004).
12. Reiling, J.H. & Hafen, E. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in *Drosophila*. *Genes Dev.* **18**, 2879–2892 (2004).
13. Sofer, A., Lei, K., Johannessen, C.M. & Ellisen, L.W. Regulation of mTOR and cell growth in response to energy stress by REDD1. *Mol. Cell. Biol.* **25**, 5834–5845 (2005).
14. Corradetti, M.N., Inoki, K. & Guan, K.L. The stress-inducted proteins RTP801 and RTP801L are negative regulators of the mammalian target of rapamycin pathway. *J. Biol. Chem.* **280**, 9769–9772 (2005).
15. Arsham, A.M., Howell, J.J. & Simon, M.C. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J. Biol. Chem.* **278**, 29655–29660 (2003).
16. Hudson, C.C. *et al.* Regulation of hypoxia-inducible factor 1α expression and function by the mammalian target of rapamycin. *Mol. Cell. Biol.* **22**, 7004–7014 (2002).
17. Katiyar, S. *et al.* REDD1, an inhibitor of mTOR signalling, is regulated by the CUL4A-DDB1 ubiquitin ligase. *EMBO Rep.* **10**, 866–872 (2009).
18. Rahman, I. *et al.* 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **166**, 490–495 (2002).
19. Rangasamy, T. *et al.* Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J. Clin. Invest.* **114**, 1248–1259 (2004).
20. Sussan, T.E. *et al.* Targeting Nrf2 with the triterpenoid CDDO-imidazolide attenuates cigarette smoke–induced emphysema and cardiac dysfunction in mice. *Proc. Natl. Acad. Sci. USA* **106**, 250–255 (2009).
21. Lin, L., Stringfield, T.M., Shi, X. & Chen, Y. Arsenite induces a cell stress-response gene, RTP801, through reactive oxygen species and transcription factors Elk-1 and CCAAT/enhancer-binding protein. *Biochem. J.* **392**, 93–102 (2005).
22. Marwick, J.A. *et al.* Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs. *Am. J. Respir. Cell Mol. Biol.* **31**, 633–642 (2004).
23. Vlahos, R. *et al.* Differential protease, innate immunity and NF-κB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290**, L931–L945 (2006).
24. Iimuro, Y. *et al.* NFκB prevents apoptosis and liver dysfunction during liver regeneration. *J. Clin. Invest.* **101**, 802–811 (1998).
25. Campbell, K.J. & Perkins, N.D. Post-translational modification of RelA(p65) NF-κB. *Biochem. Soc. Trans.* **32**, 1087–1089 (2004).
26. Zampetaki, A., Mitsialis, S.A., Pfeilschifter, J. & Kourembanas, S. Hypoxia induces macrophage inflammatory protein-2 (MIP-2) gene expression in murine macrophages via NF-κB: the prominent role of p42/ p44 and PI3 kinase pathways. *FASEB J.* **18**, 1090–1092 (2004).
27. Wikenheiser, K.A. *et al.* Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. *Proc. Natl. Acad. Sci. USA* **90**, 11029–11033 (1993).
28. DeYoung, M.P., Horak, P., Sofer, A., Sgroi, D. & Ellisen, L.W. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev.* **22**, 239–251 (2008).
29. Flotte, T.R. & Carter, B.J. Adeno-associated virus vectors for gene therapy. *Gene Ther.* **2**, 357–362 (1995).
30. Hautamaki, R.D., Kobayashi, D.K., Senior, R.M. & Shapiro, S.D. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* **277**, 2002–2004 (1997).
31. Inoki, K., Ouyang, H., Li, Y. & Guan, K.L. Signaling by target of rapamycin proteins in cell growth control. *Microbiol. Mol. Biol. Rev.* **69**, 79–100 (2005).
32. Shi, M.M., Chong, I., Godleski, J.J. & Paulauskis, J.D. Regulation of macrophage inflammatory protein-2 gene expression by oxidative stress in rat alveolar macrophages. *Immunology* **97**, 309–315 (1999).
33. Guerassimov, A. *et al.* The development of emphysema in cigarette smoke–exposed mice is strain dependent. *Am. J. Respir. Crit. Care Med.* **170**, 974–980 (2004).
34. Churg, A. *et al.* Macrophage metalloelastase mediates acute cigarette smoke–induced inflammation via tumor necrosis factor-alpha release. *Am. J. Respir. Crit. Care Med.* **167**, 1083–1089 (2003).
35. Elias, J.A., Kang, M.J., Crouthers, K., Homer, R. & Lee, C.G. State of the art. Mechanistic heterogeneity in chronic obstructive pulmonary disease: insights from transgenic mice. *Proc. Am. Thorac. Soc.* **3**, 494–498 (2006).
36. Giordano, R.J. *et al.* Targeted induction of lung endothelial cell apoptosis causes emphysema-like changes in the mouse. *J. Biol. Chem.* **283**, 29447–29460 (2008).
37. Tuder, R.M., Petrache, I., Elias, J.A., Voelkel, N.F. & Henson, P.M. Apoptosis and emphysema: the missing link. *Am. J. Respir. Cell Mol. Biol.* **28**, 551–554 (2003).
38. Gery, S. *et al.* RTP801 is a novel retinoic acid–responsive gene associated with myeloid differentiation. *Exp. Hematol.* **35**, 572–578 (2007).
39. Vandivier, R.W., Henson, P.M. & Douglas, I.S. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest* **129**, 1673–1682 (2006).
40. Lee, S.H. *et al.* Antielastin autoimmunity in tobacco smoking–induced emphysema. *Nat. Med.* **13**, 567–569 (2007).
41. Cosio, M.G., Saetta, M. & Agusti, A. Immunologic aspects of chronic obstructive pulmonary disease. *N. Engl. J. Med.* **360**, 2445–2454 (2009).
42. Rahman, I. & Adcock, I.M. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur. Respir. J.* **28**, 219–242 (2006).
43. Kang, M.J. *et al.* Cigarette smoke selectively enhances viral PAMP and viruses-induced pulmonary innate immunity and remodeling responses. *J. Clin. Invest.* **118**, 2771–2784 (2008).
44. Kim, H.P. *et al.* Autophagic proteins regulate cigarette smoke–induced apoptosis: protective role of heme oxygenase-1. *Autophagy* **4**, 887–895 (2008).
45. Gibbs, P.E. & Maines, M.D. Biliverdin inhibits activation of NF-κB: reversal of inhibition by human biliverdin reductase. *Int. J. Cancer* **121**, 2567–2574 (2007).
46. Weichhart, T. *et al.* The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity* **29**, 565–577 (2008).
47. Shah, O.J., Wang, Z. & Hunter, T. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr. Biol.* **14**, 1650–1656 (2004).
48. Bhaskar, P.T. *et al.* mTORC1 hyperactivity inhibits serum deprivation-induced apoptosis via increased hexokinase II and GLUT1 expression, sustained Mcl-1 expression, and glycogen synthase kinase 3beta inhibition. *Mol. Cell. Biol.* **29**, 5136–5147 (2009).
49. Zhang, Q., Adiseshaiah, P., Kalvakolanu, D.V. & Reddy, S.P. A phosphatidylinositol 3-kinase–regulated Akt-independent signaling promotes cigarette smoke–induced FRA-1 expression. *J. Biol. Chem.* **281**, 10174–10181 (2006).
50. Lee, D.F. *et al.* IKKβ Suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* **130**, 440–455 (2007).
51. Morelon, E., Stern, M. & Kreis, H. Interstitial pneumonitis associated with sirolimus therapy in renal-transplant recipients. *N. Engl. J. Med.* **343**, 225–226 (2000).
52. Fujitani, Y. & Trifilieff, A. *In vivo* and *in vitro* effects of SAR 943, a rapamycin analogue, on airway inflammation and remodeling. *Am. J. Respir. Crit. Care Med.* **167**, 193–198 (2003).

ONLINE METHODS

Antibodies and reagents are outlined in the **Supplementary Methods.**

Human lungs. Human normal and diseased lung tissues, which were obtained from lung transplant or lung resection donors (who gave informed consent), were processed and assessed histologically as previously described[6]. Lung samples for RT-PCR were obtained from National Jewish Health (normal controls and smokers) and from the Lung Tissue Repository Consortium (**Supplementary Table 1**). Anonymous, nontransplanted, nondiseased lung samples were obtained from Tissue Transformation Technologies. 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. The clinical severity of COPD was graded on the basis of stages defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (see **Supplementary Methods**).

Animals. Male wild-type (C57BL/6 × 129SvEv F1) (Taconic) and Rtp801-knockout mice (C57BL/6 × 129SvEv, 2 to 6 months old) (generated by Lexicon Genetics for Quark Pharmaceuticals.) were used[10]. All experiments conducted in mice were approved by the Animal Care Use Committees of Johns Hopkins University, Harvard University and University of Colorado.

Cigarette smoke exposure. Mice were exposed to room air as a control or exposed to cigarette smoke (n = 3–7 mice in each group) as previously described[19]. The antioxidant NAC (500 mg per kg body weight) (Sigma) was intraperitoneally injected into wild-type mice twice (day 0 and 1) before initiation of cigarette smoke exposure for 1 d, and mice were killed just after the exposure. Six-month exposures to cigarette smoke were performed as previously described[30]. Rtp801 mRNA expression was also investigated in lung samples from C57BL/6 wild-type and *Nfe2l2*$^{-/-}$ mice exposed to 4–6 months of cigarette smoke[20].

AAV5-*DDIT4* constructs and administration to mice. *Cre* and human *DDIT4* cDNAs were cloned into an intermediate vector flanked by AAV2 internal inverted repeats. Expression was confirmed by western blot analysis of transfected HEK293 cells. AAV5-*DDIT4* and AAV5-*Cre* viruses were prepared as previously described[53]. Wild-type mice received 2×10^{10} of AAV5-*DDIT4* or AAV5-*Cre* viral particles via intratracheal instillation. The experiments with *DDIT4, DDIT*-RPAA and pcDNA3 *in vivo* instillation are described in the **Supplementary Methods**.

IκBα superrepressor experiments. For *in vivo* experiments, wild-type (C57BL/6 × 129SvEv or C57BL/6/J mice as LPS controls; 2 months old) were instilled intratracheally with 1×10^9 plaque-forming units of adenovirus-5 (Ad 5) encoding IκB superrepressor or Ad5 encoding LacZ (in 50 µl of sterile PBS). Seven to ten days later, the mice were exposed to cigarette smoke. Controls consisted of room air–exposed mice. LPS was instilled in 50 µl sterile PBS (30 µg ml^{-1}) and killed 16 h later. For *in vitro* experiments, A549 cells were infected with an adenovirus construct containing superrepressor IκB or LacZ at approximately 10 plaque-forming units per cell. Twenty-four to thirty-six hours later, we added 2% or 5% CSE for 6 h (defined in preliminary studies with concentrations varying from 1% to 10% for 6, 12, 18 and 24 h) or tumor necrosis factor-α (10 ng ml^{-1}) for 1 h as positive control.

Rapamycin experiments. For *in vivo* experiments, 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 per kg body weight) or vehicle (1.8% DMSO) to wild-type and Rtp801–knockout mice for 7 d before exposure to cigarette smoke for 4 d or room air. For *in vitro* experiments, MLFs were prepared as described in the **Supplementary Methods** and were pretreated with 10 nM of rapamycin for 3 d before CSE exposure and then treated with 1% CSE for the periods indicated in the legends of **Figure 6** and **Supplementary Figure 5**.

Cell culture and cigarette smoke extract preparation. MLFs and MEFs were isolated from wild-type and Rtp801-knockout 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 third and seventh passages, whereas rat lung microvascular endothelial cells and Beas2B cells were used up to passage 20. CSE was prepared as described previously[6].

***DDIT4* and nuclear favtor-κB complex promoter assays.** For *DDIT4* promoter assay, MLFs, HEK293 cells and Beas2B cells were transfected with firefly luciferase expression vectors of the full-length *DDIT4* 2.5-kb promoter or with a point mutation within the C/EBP binding site or with an empty vector (pGL3 basic)[21]. Two independent experiments were performed each in triplicate (the s.d. 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-κB complex promoter assay, approximately 8 to 11 × 10^4 MLFs and rat lung microvascular endothelial cells were transfected with pHTS NF-κB reporter vector encoding firefly luciferase, *DDIT4* (779 bp) plasmid DNA, both, or pcDNA3.1 (+). Transfection conditions are detailed in the **Supplementary Methods**.

Additional methodology. 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 analyses. Data are represented as means ± s.e.m. Multiple comparison analyses were done by ANOVA with Tukey's *post hoc* test or Kruskal-Wallis nonparametric analysis of variance with Dunnet's T_3 *post hoc* 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.

53. Petrache, I. *et al.* α-1 antitrypsin inhibits caspase-3 activity, preventing lung endothelial cell apoptosis. *Am. J. Pathol.* **169**, 1155–1166 (2006).
54. Neuhaus, P., Klupp, J. & Langrehr, J.M. mTOR inhibitors: an overview. *Liver Transpl.* **7**, 473–484 (2001).
55. Faivre, S., Kroemer, G. & Raymond, E. Current development of mTOR inhibitors as anticancer agents. *Nat. Rev. Drug Discov.* **5**, 671–688 (2006).
56. McGrath-Morrow, S.A. *et al.* Vascular endothelial growth factor receptor 2 blockade disrupts postnatal lung development. *Am. J. Respir. Cell Mol. Biol.* **32**, 420–427 (2005).

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