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Cathepsin E Promotes Pulmonary Emphysema via Mitochondrial Fission

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Emphysema is characterized by loss of lung elasticity and irreversible air space enlargement, usually in the later decades of life. The molecular mechanisms of emphysema remain poorly defined. We identified a role for a novel cathepsin, cathepsin E, in promoting emphysema by inducing mitochondrial fission. Unlike previously reported cysteine cathepsins, which have been implicated in cigarette smoke-induced lung disease, cathepsin E is a nonlysosomal intracellular aspartic protease whose function has been described only in antigen processing. We examined lung tissue sections of persons with chronic obstructive pulmonary disease, a clinical entity that includes emphysematous change. Human chronic obstructive pulmonary disease lungs had markedly increased cathepsin E protein in the lung epithelium. We generated lung epithelial-targeted transgenic cathepsin E mice and found that they develop emphysema. Overexpression of cathepsin E resulted in increased E3 ubiquitin ligase parkin, mitochondrial fission protein dynamin-related protein 1, caspase activation/apoptosis, and ultimately loss of lung parenchyma resembling emphysema. Inhibiting dynamin-related protein 1, using a small molecule inhibitor *in vitro* or *in vivo*, inhibited cathepsin E-induced apoptosis and emphysema. To the best of our knowledge, our study is the first to identify links between cathepsin E, mitochondrial fission, and caspase activation/apoptosis in the pathogenesis of pulmonary emphysema. Our data expand the current understanding of molecular mechanisms of emphysema development and may provide new therapeutic targets. *(Am J Pathol 2014, 184: 2730–2741; http://dx.doi.org/10.1016/j.ajpath.2014.06.017)*

Emphysema is a major subset of chronic obstructive pulmonary disease (COPD) and is defined anatomically as the destruction of the distal lung parenchyma and enlargement of the air spaces. Pulmonary emphysema is one of the main causes of morbidity and death worldwide. The most studied factor in developing COPD has long been recognized to be cigarette smoking. However, only 10% to 20% of heavy smokers develop clinically significant COPD.[1,2] Importantly, recent studies indicate that complementary pathogenic mechanisms, such as proteolytic/antiproteolytic imbalance, oxidative stress, apoptosis, or altered innate immunity, are involved in the development and progression of alveolar destruction.[3–6]

Cathepsins have been implicated in mediating alveolar destruction via their proteolytic activity. Cathepsins are intracellular hydrolases and include serine proteases (cathepsins A and G), aspartic proteases (cathepsins D and E), and cysteine cathepsins (cathepsins B, C, F, H, K, L, O, S, V, X, and W). Cathepsin E (Cat E), a nonlysosomal intracellular aspartic protease, is homologous to aspartic protease cathepsin D, a major proteolytic activity in the lysosomal component.[7] Recent studies have reported that Cat E plays an important role in antigen processing via the major histocompatibility complex class II pathway, host defense against cancer cells and invading microorganisms, gastric differentiation, and development of signet-ring cell carcinoma.[8–12] However, Cat E has not been linked to lung disease.

Human lung sections from persons with COPD indicated increased expression of Cat E protein in the lung epithelial cells. To investigate if increased expression of lung epithelial

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Cat E could lead to emphysema, we generated lung-targeted constitutive and inducible Cat E transgenic (Tg) mice. Our data indicated that inducible Cat E Tg mice developed emphysema-like lung changes as early as 1 week. We noted robust caspase 3 activation, and, when mice were administered a caspase inhibitor, emphysema was prevented. To our surprise, we did not find changes in caspases usually associated with caspase 3 activation, such as caspases 8 and 9, in Cat E Tg mice. Instead, we found significant induction of a mitochondrial fission protein, dynamin-related protein 1 (Drp1). When we inhibited Drp1 in Cat E Tg mice with Mdivi-1, a small molecule Drp1 inhibitor, we completely abolished the development of emphysema. Collectively, our data indicate that increased Cat E is a clinically relevant finding in human COPD and invoke a novel role for Cat E in mitochondrial fission-induced emphysema.

Materials and Methods

Animals

The CC10-Cat E constitutive Tg mice were generated by cloning the human Cat E cDNA (Open Biosystems, Lafayette, CO) downstream of the rat Clara cell 10-kDa protein (CC10) promoter and upstream of human growth hormone (hGH) polyadenylation and intronic sequence. Two copies of the chicken β-globin insulator sequence were flanked on both ends of the transgene cassette (Figure 1A). Tg mice were generated in (C57BL/6 × SJL/J) F2 eggs by using standard pronuclear injection and backcrossed onto the C57BL/6 background for >10 generations before use. The transgene was genotyped via PCR with the following transgene-specific primers: 5′-ACACGCATACCCACACATAC-3′ and 5′-TACTGGAGTCACTCCTCCCA-3′.

For generating inducible Cat E Tg mice, constructs that contained the *CC10* promoter, the reverse tetracycline transactivator, hGH intronic and polyadenylation sequences and a polymeric tetracycline operator; a minimal cytomegalovirus promoter, human Cat E cDNA, and hGH were used (Figure 1B). Tg mice were generated in (C57BL/6 × SJL/J) F2 eggs by using standard pronuclear injection and backcrossed onto the C57BL/6 background for >10 generations before use. All mice were evaluated for the presence of both reverse tetracycline transactivator and Cat E with the use of PCR analysis. PCR for Cat E used the following primers: sense 5′-TCCCCTCTGTGTACTGCACT-3′ and antisense 5′-TACTGGAGTCACTCCTCCCA-3′.

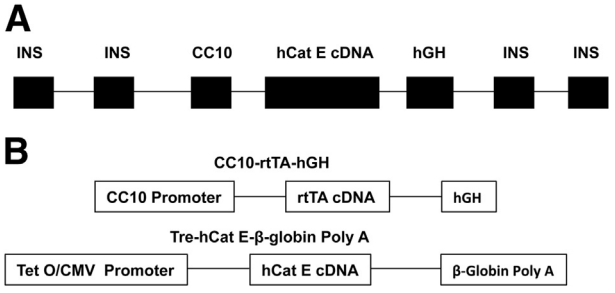

Figure 1 Generation of lung epithelial-targeted constitutive Cat E Tg mice. **A:** Construct for generation of lung epithelial-targeted, constitutive CC10-Cat E Tg mice. **B:** Construct for generation of inducible CC10-Cat E Tg mouse. CMV, cytomegalovirus; hCat E, human Cat E; INS, insulator; rtTA, reverse tetracycline transactivator; Tet O, tetracycline operator.

In experiments performed with inducible Cat E Tg mice and their littermate wild-type (WT) controls, all mice were maintained on normal water until they were 1 month old. They were then randomly assigned to receive either normal water or water with doxycycline (dox) for the duration of the experiment. Dox was administered at 500 mg/L in 4% sucrose and kept in dark bottles to prevent light-induced degradation. Mice were maintained under specific pathogen-free conditions at the animal facility of Yale University School of Medicine. Animal protocols were reviewed and approved by the Animal Care and Use Committee at Yale University.

Administration of Mdivi-1 and Z-VAD

Small molecule Drp1 inhibitor, Mdivi-1 (50 mg/kg body weight/day; Enzo Life Sciences, Farmingdale, NY) and broad caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD; 3 mg/kg body weight/day; Calbiochem, La Jolla, CA) were administered to Cat E Tg mice and WT littermates by intraperitoneal injection. Mdivi-1 or Z-VAD was given 1 day before dox water and was repeated daily for 2 weeks.

Exposure to Cigarette Smoke

Eight-week-old C57BL/6J mice were exposed to room air or the smoke from nonfiltered research cigarettes (2R4; University of Kentucky, Lexington, KY) as previously described.[13] During the first week, mice received half a cigarette twice a day to allow for acclimation, and then two cigarettes per day (one cigarette per session, two sessions per day) for up to 6 months.

Bronchoalveolar Lavage and Lung Inflammation

Lung inflammation was assessed by bronchoalveolar lavage, as described previously.[14] In brief, animals were anesthetized, a median sternotomy was performed, the trachea was dissected free from the underlying soft tissues, and bronchoalveolar lavage was performed by perfusing the lungs *in situ* with 0.9 mL of phosphate-buffered saline and gently aspirating the fluid back. This procedure was repeated twice. Samples were then pooled and centrifuged, and total cell numbers and differentials were assessed. The cell-free bronchoalveolar lavage fluid was stored at −70°C until used.

Lung Volume, Histological Evaluation, and Morphometric Analysis

Lung volume was assessed as previously described.[6,15] In brief, mice were anesthetized, the trachea was cannulated, and the lungs were removed and inflated with phosphate-buffered saline at 25 cm H_2O. The size of the lung was evaluated by volume displacement. Hematoxylin and eosin staining was performed after pressure fixation with 10% buffered formalin solution in the Research Pathology Laboratory at Yale University. Alveolar size was estimated from the mean chord length of the air space as previously described.[6,15]

Immunofluorescence and Immunohistochemistry

Formalin-fixed, paraffin-embedded lung tissue sections from human (COPD and non-COPD) and mouse lungs were analyzed for Cat E and surfactant protein C immunofluorescence and immunohistochemical staining as described previously.[16,17] Cat E (dilution 1:400) and surfactant protein C (dilution 1:200) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

MLE12 cells, a murine lung epithelial cell line, were purchased from ATCC (Manassas, VA) and cultured as previously described.[18] For Mdivi-1 treatment, MLE12 cells were treated with Mdivi-1 (50 μmol/L) 30 minutes before treatment with activated Cat E (100 ng/mL). Human Cat E-overexpressing plasmid (pCC10-Cat E), empty vector, or *Drp1K38A,* a dominant-negative mutant of Drp1, was transfected into MLE12 cells as previously described[19] by using Lipofactamine 2000 (Invitrogen, Carlsbad, CA). After incubation, cells were cultured for an additional 48 hours in complete medium.

Measurement of Caspase 3, 8, and 9 Activities

Caspase 3, 8, and 9 activities were measured according to the manufacturer's instructions and as previously described.[18]

Apoptosis Assays

Terminal deoxynucleotidyltransferase-dUTP nick end-labeling (TUNEL) and flow cytometric assays were used as previously described.[20]

Western Blot Analysis

Protein was extracted from cell or lung tissue lysates, electrotransferred, and then immunoblotted with rabbit Cat E (Santa Cruz Biotechnology), rabbit caspase 3 (Santa Cruz Biotechnology), or rabbit Drp1 (Abcam, Cambridge, MA) antibodies. Detection was performed with Phototope-HRP Western Blot Detection System (Cell Signaling Technology, Danvers, MA). Equivalent sample loading was confirmed by stripping membranes with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and reprobing with anti−β-tubulin or anti−β-actin antibodies (Santa Cruz Biotechnology).

RNA Extraction and mRNA Analyses

Total RNA extraction and mRNA analyses (reverse transcription-PCR or real-time RT-PCR) were described previously.[6,21] Primers used for mouse Cat E were sense, 5′-TCCCAGACACCGCTCCTCCAG-3′, and antisense, 5′-GGATTTGGGGCTGCTGGTTTC-3′; Parkin sense, 5′-TGGGAGGTGTGCTGTGCCCCCG-3′, and antisense, 5′-AAAACAAACCCGCAGCCCAGGCCGT-3′; Drp1 sense, 5′-CAGGAATTGTTACGGTTCCCTAA-3′, and antisense, 5′-CCTGAATTAACTTGTCCCGTGA-3′; fission protein 1 (Fis1) sense, 5′-AGAGCACGCAATTTGAATATGCC-3′, and antisense, 5′-ATAGTCCCGCTGTTCCTCTTT-3′; mitofusin 1 (Mfn1) sense, 5′-AACTTGATCGAATAGCATCCGAG-3′, and antisense, 5′-GCATTGCATTGATGACAGAGC-3′; mitofusin 2 (Mfn2) sense, 5′-CTGGGGACCGGATCTTCTTC-3′, and antisense, 5′-CTGCCTCTCGAAATTCTGAAACT-3′; optic atrophy 1 (Opa1) sense, 5′-CGACTTTGCCGAGGATAGCTT-3′, and antisense, 5′-CGTTGTGAACACACTGCTCTTG-3′; and for mouse glyceraldehyde-3-phosphate dehydrogenase, sense, 5′-TGTGTCCGTCGTGGATCTGA-3′, and antisense, 5′-CCTGCTTCACCACCTTCTTGAT-3′.

Measurement of Cat E Enzymatic Activity

Enzymatic activity of lung tissue Cat E was measured as described,[22] with some modifications. Briefly, a total volume of 100 μL of reaction mixture that contained 80 μL of buffer (50 mmol/L of sodium acetate buffer, pH 4.0), 10 μL of 200 mmol/L substrate solution, and 10 μL of sample solution was incubated at 40°C for 10 minutes, and the reaction was terminated by adding 2 mL of 5% trichloroacetic acid. The increase in fluorescence intensity produced by substrate cleavage during incubation was measured at an emission wavelength of 393 nm with excitation at 328 nm by using a fluorescence spectrophotometer. The recombinant Cat E standard was purchased from R&D Systems (Minneapolis, MN). The Cat E hydrolysis fluorogenic substrate MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys (Dnp)-D-Arg-NH2 (KYS-1) was purchased from Peptide International Inc. (Louisville, KY).

Preparation of siRNA and Transfection of siRNA Duplexes

Mouse Cat E siRNA targeting sequence NNCGAGUCCUGUAAUGUGUAU was synthesized in 2′-deprotected, duplexed, desalted, and purified form by Dharmacon Research Inc. (Lafayette, CO). The sense and antisense strands of mouse Cat E siRNA were sense, 5′-CGAGUCCUGUAAUGUGUAUdTdT-3′, and antisense, 5′-AUACACAUUACAGGA-

CUCGdTdT-3′. Parkin siRNA was purchased from Santa Cruz Biotechnology Inc. Nonspecific siRNA scrambled duplex probes (sense, 5′-GCGCGCUUUGUAGGAUUCG-3′; antisense, 5′-CGAAUCCUACAAAGCGCGC-3′) were synthesized by Dharmacon Research Inc. as previously described.[6,20] Transfection of siRNA duplexes was described previously.[6,19,20]

Measurement of Proteasome 20S Activity

The proteasome 20S activity was evaluated in whole-cell lysate by means of a proteasome 20S activity kit (APT 280; Millipore, Billerica, MA), as described by the manufacturer. Whole-cell lysate protein extract (10 μg) was incubated in the provided buffer with 3.8 mg of fluorophore-linked peptide substrate (LLVY-7-amino-4-methylcoumarin) for 120 minutes at 37°C. Proteasome activity was measured by quantification of relative fluorescent units from the release of the fluorescent-cleaved product LLVY-7-amino-4-methylcoumarin by using a 380/460-nm filter. A solution of the proteasome 20S subunit and the proteasome inhibitor lactacystin were used as controls for the assay.

Assessment of Mitochondrial Morphology

Cultured MLE12 cells were treated with or without activated Cat E (100 ng/mL) for 16 hours, and then the cells were processed for mitochondrial immunofluorescence or transmission electron microscopy studies. For mitochondrial immunofluorescence studies, the cells were stained with 1:2000 diluted MitoTracker Red CMX Ros Mitochondrial Probe (Invitrogen) at 37°C for 15 minutes. After washing, the cells were fixed with 3.7% formaldehyde and counterstained with DAPI mounting solution. For transmission electron microscopy studies, the cells were fixed with 2% (v/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde in 0.1 mol/L sodium cacodylate, postfixed with 1% (w/v) osmium tetroxide, followed by 1% (w/v) uranyl acetate, dehydrated, and embedded in LX112 resin. Ultrathin sections stained with uranyl acetate followed by lead citrate were viewed on a FEI Tecnai transmission electron microscope (Hillsboro, OR).

Statistical Analysis

Data are expressed as means ± SEM and were analyzed by two-tailed Student's *t*-test. $P < 0.05$ was considered to be significant.

Results

Increased Cat E in Human COPD and Smoking Mouse Lungs

To determine the clinical relevance of Cat E in COPD, we performed immunohistochemistry for Cat E on 10 lung sections obtained from former smokers with COPD (as defined by criteria of the global initiative for COPD) and on 6 lung sections obtained from nonsmokers without COPD, provided by the Lung Pathology Department of Yale University School of Medicine. COPD lung sections had significantly increased Cat E staining compared with lung sections without COPD (Figure 2, A–C). COPD lungs had increased lung epithelial Cat E staining compared with the weak and scattered staining in non-COPD lungs. Given that smoking can cause COPD, smoke-exposed mice were used to study the pathogenesis of COPD. We investigated whether smoking can induce Cat E expression in mouse lungs. Mouse lungs showed a significant Cat E mRNA expression after 6 months of smoking, a time point commonly associated with COPD-like changes in mouse lungs (Figure 2D).

Cat E Overexpression Results in Emphysema

To determine whether increased Cat E in the lung causes emphysema, we generated lung epithelial-specific, Tg Cat E overexpression mice. We first generated constitutively expressed lung epithelial-specific Tg mice to determine whether any effect was on lung architecture by Cat E induction. We first confirmed increased Cat E protein expression in lungs by using immunohistochemistry (Figure 3A) and Western blot analysis (Figure 3B). We also observed increased Cat E enzymatic activity in Cat E Tg lung tissues (Figure 3C). By 1 month, constitutive Cat E Tg mice showed histological lung changes consistent with

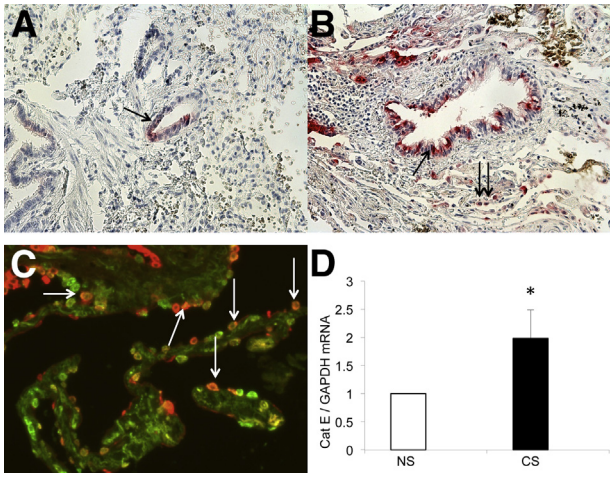

Figure 2 Human COPD lung section and smoking mouse lungs showed increased Cat E expression. Representative immunohistochemical staining for Cat E in human lung tissue with (**B**) or without (**A**) COPD. Cat E protein is detected as red cytoplasmic staining (**single arrow**, airway epithelium; **double arrow**, lung alveolar epithelial cells). **C:** Cat E expression in human COPD lung alveolar epithelial cells [Cat E (red); SP-C (green); **arrows**, alveolar epithelial cells with Cat E expression]. **D:** Cat E expression is increased in mouse lungs after 6 months of CS compared with NS lungs. Data are expressed as means ± SEM. $*P < 0.05$ versus mouse NS lungs. Original magnification: ×200 (**A** and **B**); ×400 (**C**). CS, cigarette smoke; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, without smoking; SP-C, surfactant protein C.

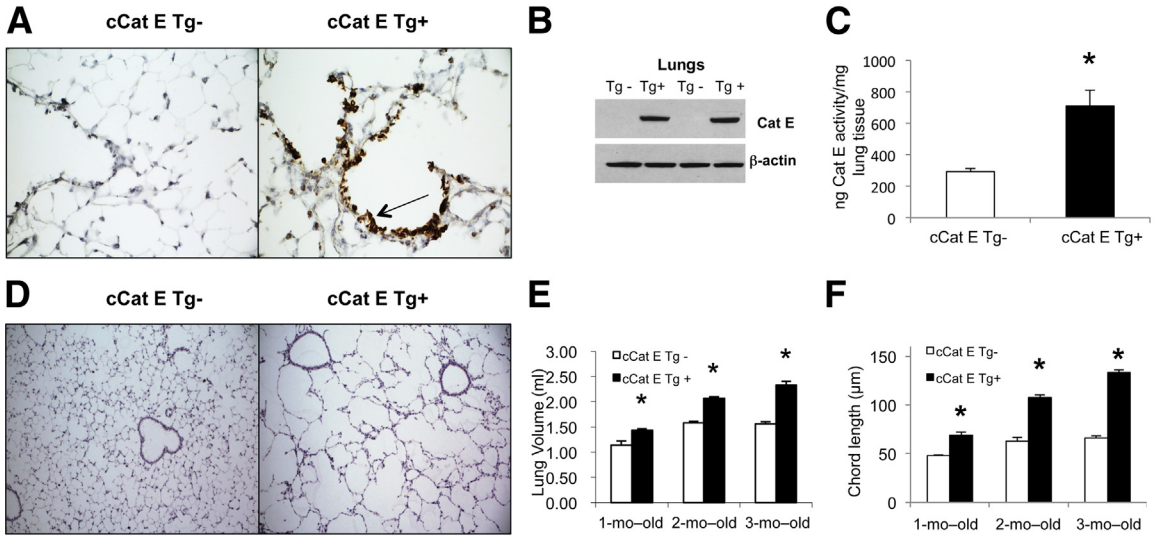

Figure 3 Lung epithelial-targeted constitutive Cat E Tg mice develop emphysema. **A:** Cat E protein expression in cCat E Tg^+ mouse lungs and Tg^- WT littermates detected by immunohistochemistry (**arrow**, positive brown staining). **B:** Cat E protein expression in cCat E Tg^+ mouse lungs compared with Tg^- mouse lungs detected by Western blot analysis. **C:** cCat E Tg^+ mouse lungs showed increased Cat E enzymatic activity compared with Tg^- WT littermates. **D:** Representative lung histology of 3-month-old cCat E Tg^+ mouse lungs with emphysema versus WT littermates. **E:** cCat E Tg^+ and Tg^- mouse lung volumes at different ages. **F:** cCat E Tg^+ and Tg^- mouse lung mean linear chord lengths at different ages. Data are expressed as means ± SEM. $*P < 0.05$ versus cCat E Tg^- WT littermates. Original magnification: ×400 (**A**); ×100 (**D**). cCat E, constitutive CC10-Cat E.

emphysema, as characterized by enlargement of the air spaces distal to the terminal bronchioles and loss of the normal alveolar architecture (Figure 3D). These changes were accompanied by increased lung volumes (Figure 3E) and mean linear chord lengths (Figure 3F).

We then generated dox-inducible Cat E Tg mice to regulate Cat E postnatally. Inducible Cat E Tg mice and WT littermates were randomly assigned to receive normal water or dox-containing water, starting at 1 month of age. Similar to constitutive Tg mice, inducible Tg mice showed increased Cat E protein expression by immunofluorescence staining (Figure 4A), Western blot analysis (Figure 4B), and Cat E enzymatic activity (Figure 4C). Inducible Cat E Tg mice also showed histological evidence of emphysema, increased lung volumes, and increased mean linear chord lengths (Figure 4, D–F). We used inducible Tg mice and

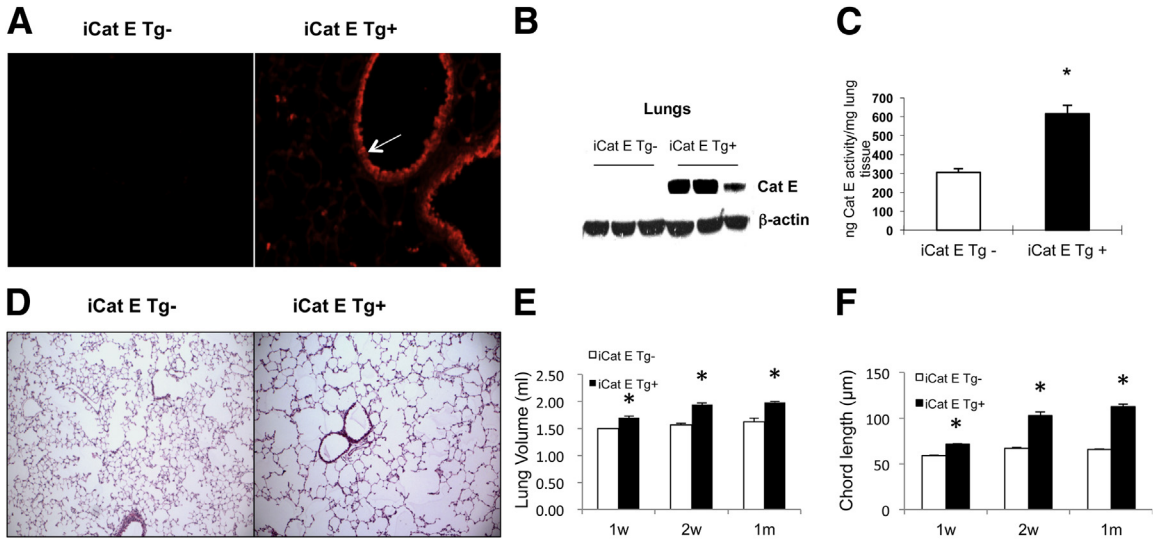

Figure 4 Lung epithelial-targeted inducible Cat E Tg mice develop emphysema. **A:** Cat E protein expression in iCat E Tg^+ mouse lungs (after 2 weeks of dox water) and Tg^- WT littermates detected by immunofluorescence (**arrow**, positive red staining). **B:** Cat E protein expression in iCat E Tg^+ mouse lungs after 2 weeks of dox water compared with Tg^- mouse lungs detected by Western blot analysis. **C:** iCat E Tg^+ mouse lungs showed increased Cat E enzymatic activity compared with Cat E Tg^- WT littermates. **D:** Representative lung histology of iCat E Tg^+ mouse lungs (after 2 weeks of dox water) with emphysema versus WT littermates. **E:** iCat E Tg^+ and Tg^- mouse lung volumes after a time course of dox water. **F:** iCat E Tg^+ and Tg^- mouse lung mean linear chord lengths at different time points after a time course of dox water. Data are expressed as means ± SEM. $*P < 0.05$ versus iCat E Tg^- WT littermates. Original magnification: ×200 (**A**); ×100 (**D**). iCat E, inducible CC10-Cat E.

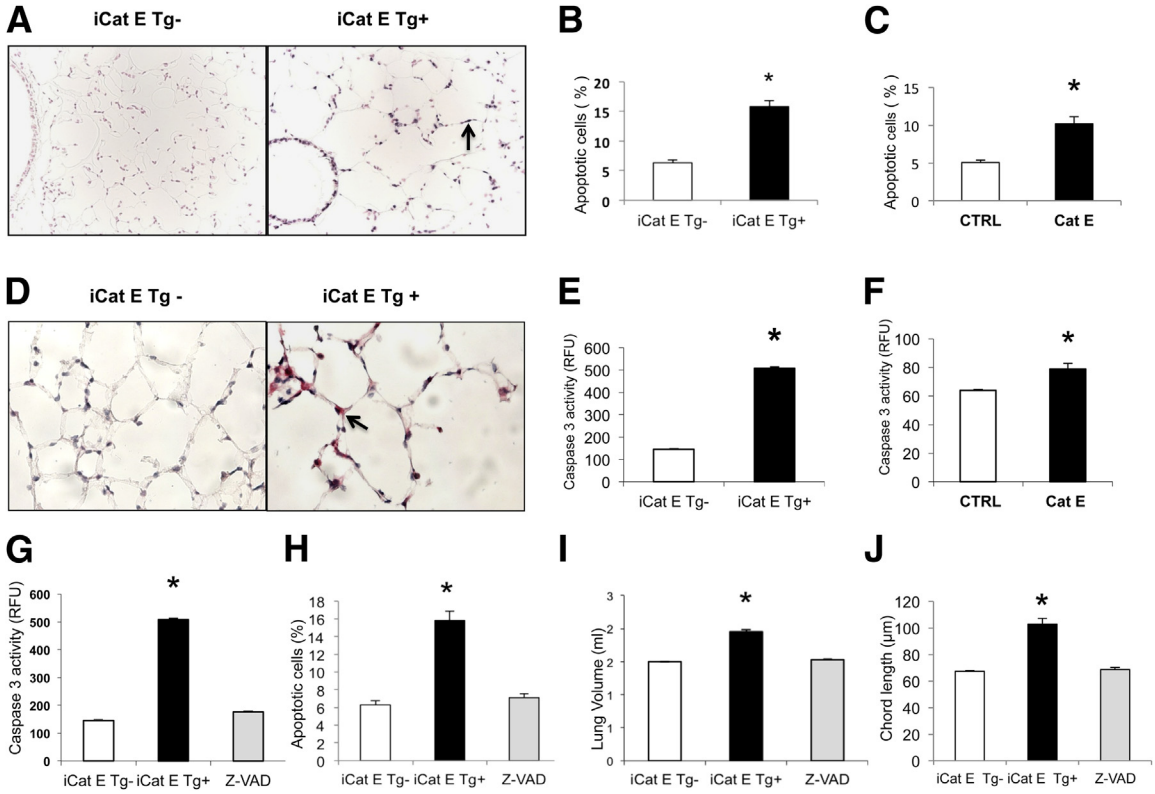

Figure 5 Cat E causes caspase 3-mediated apoptosis, and caspase inhibition prevents the development of emphysema. **A:** Representative sections from iCat E Tg^+ mouse lung stained for TUNEL after 2 weeks of dox water showed increased TUNEL positivity compared with Tg^- littermate WT mouse lung (TUNEL staining; **arrow**, nuclear blue staining). **B:** Quantitation of TUNEL-positive cells is expressed as percentage of total cells in lung sections. **C:** MLE12 cells were treated with 100 ng/mL human recombinant active Cat E for 48 hours and apoptosis was analyzed by flow cytometry. **D:** Representative caspase 3 activation detected by immunohistochemical staining in iCat E Tg^+ mouse lung compared with Tg^- littermate WT mouse lung (**arrow**, positive red cytoplasmic staining for active caspase 3). **E:** iCat E Tg^+ mouse lungs showed increased caspase 3 activity. **F:** MLE12 cells treated with recombinant active Cat E (100 ng/mL) for 48 hours showed increased caspase 3 activity. Z-VAD, a broad caspase inhibitor, was administered 1 day before dox water and repeated daily for 2 weeks via intraperitoneal injection (3 mg/kg body weight/day). Z-VAD treatment decreased lung caspase 3 activity in iCat E Tg^+ mouse lungs (**G**), TUNEL-positive apoptotic cells in iCat E Tg^+ mouse lungs (**H**), lung volume in iCat E Tg^+ mouse lungs (**I**), and lung emphysema in iCat E Tg^+ mice, as measured by mean linear chord length (**J**). Data are expressed as means ± SEM (**B, C,** and **E**–**J**). *$P < 0.05$ versus MLE12 cells without active Cat E treatment CTRL (**C** and **F**), versus iCat E Tg^- WT littermates (**B** and **E**), versus iCat E Tg^+ mice treated with Z-VAD or iCat E Tg^- WT littermates (**G**–**J**). Original magnification: ×400 (**A**); ×600 (**D**). CTRL, control; iCat E, inducible CC10-Cat E; RFU, relative fluorescence unit.

WT littermates for the subsequent studies. Of note, neither constitutive nor inducible Cat E Tg mouse lungs exhibited inflammatory cell influx (data not shown).

Inhibiting Apoptosis Prevents Cat E-Induced Emphysema

Our previous work and other studies have found that apoptosis plays an important role in the pathogenesis of emphysema in animal models and humans.[4,6,23] Therefore, we evaluated apoptosis in Cat E Tg mice by TUNEL staining and found an increased number of TUNEL-positive cells in the lungs of Cat E Tg mice compared with WT lungs (Figure 5, A and B). We confirmed our results by using flow cytometric quantitation of annexin V–propidium iodide staining in MLE12 cells, which exhibited significantly increased apoptosis after treatment with recombinant, activated Cat E compared with control cells without Cat E treatment (Figure 5C). Next, we asked whether caspase 3-mediated cell death existed in Cat E Tg mouse lungs and found a significant increase in activated caspase 3-positive cells (Figure 5D) and caspase 3 activity (Figure 5E) in Cat E Tg mouse lungs. Similarly, in MLE12 cells we also identified increased caspase 3 activity and cleavage after treatment with recombinant active Cat E compared with control cells (Figure 5F). However, we did not find differences in caspase 8 or caspase 9 activation in Cat E Tg mouse lungs or Cat E-treated MLE12 cell. To determine whether increased caspase 3 activity is a causative factor in developing emphysema, we used a broad caspase inhibitor, Z-VAD. Cat E Tg mice treated with Z-VAD showed a significant decrease in caspase 3 activity and apoptosis (Figure 5, G and H). Z-VAD also prevented emphysema, as shown by decreased lung volume (Figure 5I) and mean linear chord length (Figure 5J). Our data indicated that Cat E overexpression leads to increased caspase 3-mediated cell death and emphysema.

Cat E Overexpression Induces Mitochondrial Fission

Recent studies have found that proteins regulating mitochondrial fission and fusion dynamics are associated with a broad range of cellular functions and disease-related processes such as apoptosis, aging, chronic heart diseases, diabetes, or neuron-degenerative diseases.[24–30] However, the association of mitochondrial dynamics and pulmonary emphysema was not clear. We first investigated if there

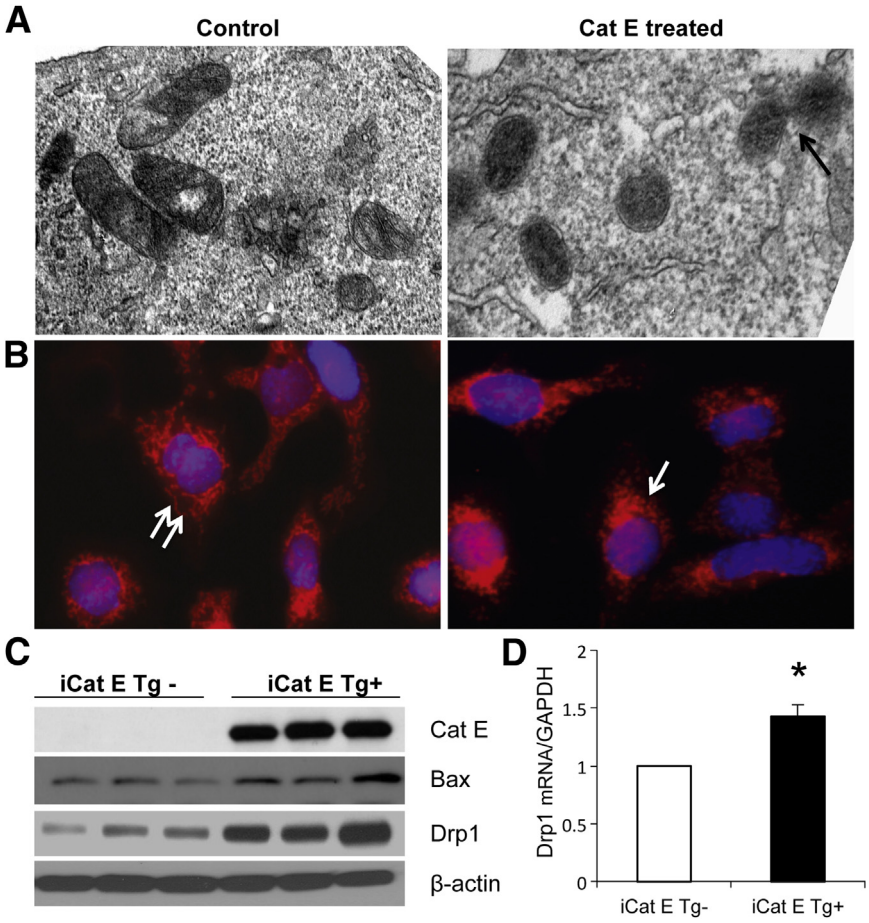

Figure 6 Cat E induces mitochondria fission and mitochondrial fission protein Drp1. **A:** MLE12 cells were treated with 100 ng/mL human recombinant active Cat E for 16 hours, and mitochondrial morphology was observed by transmission electron microscope. Cells treated with Cat E showed disorganized, smaller, and fragmented mitochondria compared with control cells (**arrow**, mitochondrion undergoing fission). **B:** MLE12 cells were treated with 100 ng/mL human recombinant active Cat E for 16 hours, and mitochondria morphology was observed by mitochondrial immunofluorescence. Cells treated with active Cat E showed disorganized, smaller, and fragmented mitochondria (**single arrow**, fragmented mitochondria) compared with control cells (**double arrow**, elongated mitochondria). **C:** iCat E Tg^+ mouse lungs (after 2 weeks of dox water) showed increased Drp1 fission protein and proapoptotic Bax expression by Western blot analysis. **D:** iCat E Tg^+ mouse lungs showed increased Drp1 mRNA expression detected by real-time reverse transcription PCR. Data are expressed as means ± SEM. $^*P < 0.05$ versus iCat E Tg^- WT littermates. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iCat E, inducible CC10-Cat E.

were changes in mitochondrial morphology to indicate mitochondrial fusion or fission by using mitochondrial immunofluorescence and transmission electron microscopy studies. Compared with control groups, Cat E-treated MLE12 cells showed disorganized, smaller, and fragmented mitochondria by mitochondrial immunofluorescence and transmission electron microscopy studies, indicating increased mitochondrial fission (Figure 6, A and B). We then studied if changes in proteins were associated with mitochondrial fission and fusion and found fission protein Drp1 was significantly increased at protein and mRNA levels in Cat E Tg mouse lungs compared with WT littermates (Figure 6, C and D). No differences were found in other fission proteins such as Fis1 and in fusion proteins such as Opa1, Mfn1, and Mfn2 between lungs of Cat E Tg and WT mice (data not shown).

Inhibition of Mitochondrial Fission Protein Drp1 Decreases Caspase 3 Activity and Prevents Emphysema in Cat E Tg Mice

Mitochondrial fission and fusion have important roles in regulating cell apoptosis.[31] Drp1 has been reported to colocalize with Bax in mitochondria during apoptosis. We identified increased Drp1 expression and Bax expression in Cat E Tg mouse lungs (Figure 6C). Next, we investigated if the increased mitochondrial fission mediator Drp1 is involved in caspase 3 activation, apoptosis, and developing emphysema *in vitro* or *in vivo*. Our data indicated that inhibition of Drp1 with the highly specific small molecule Drp1 inhibitor, Mdivi-1,[24,32] inhibited caspase 3 cleavage/activation in MLE12 cells treated with recombinant active Cat E (Figure 7A) and caspase 3 activity in Cat E Tg mouse lungs (Figure 7B). We also found that inhibition of Drp1 with *Drp1K38A*,[33,34] a dominant-negative mutant of Drp1, decreased apoptosis in MLE12 cells transfected with Cat E overexpressing plasmid (Figure 7C). Mdivi-1 administration *in vivo* decreased apoptosis (Figure 7D) and ameliorated emphysema in inducible Cat E Tg mice (Figure 7, E–G). These data suggested that mitochondrial fission regulates caspase 3-mediated apoptosis in the lung and that inhibition of mitochondrial fission protein Drp1 will prevent apoptosis and emphysema.

Parkin Is Involved in Cat E-Regulated Mitochondrial Fission and Drp1 Expression

Parkin, an E3 ubiquitin ligase, plays a critical role in regulating mitochondrial fission and fusion.[35] Recent studies have suggested that Parkin interacts with proteins that regulate

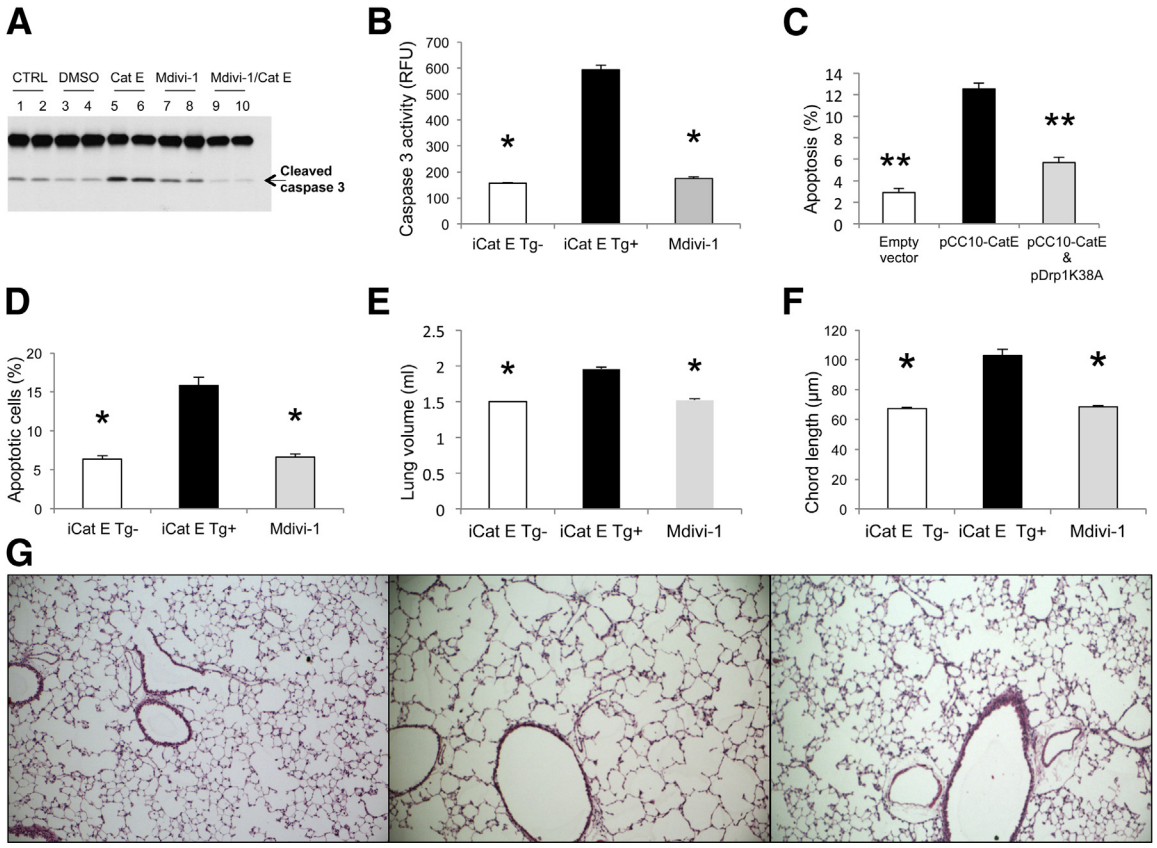

Figure 7 Inhibiting Drp1 prevents Cat E-induced emphysema. **A:** MLE12 cells treated with 100 ng/mL human recombinant active Cat E showed increased activation of caspase 3 by demonstrating caspase 3 cleavage detected by Western blot analysis (lanes 5 and 6). Mdivi-1 was administered 1 day before dox water and repeated daily for 2 weeks via intraperitoneal injection (50 mg/kg body weight/day). **B:** Mdivi-1 treatment inhibits caspase 3 activity in iCat E Tg^+ mouse lungs. **C:** MLE12 cells were transfected with pCC10-Cat E, empty vector, or *Drp1K38A*, a dominant-negative mutant of Drp1. After incubation, cells were cultured for an additional 48 hours, and apoptosis was analyzed by flow cytometry. **D:** Mdivi-1 treatment significantly decreases TUNEL-positive lung cells in iCat E Tg^+ mouse lungs. **E:** Mdivi-1 treatment decreases lung volumes in iCat E Tg^+ mice. **F:** Mdivi-1 treatment decreases lung emphysema in iCat E Tg^+ mice, as measured by mean linear chord length. **G:** Representative lung histology of iCat E Tg^+ mouse lungs with (**right panel**) or without (**middle panel**) Mdivi-1 treatment versus WT littermates (**left panel**). Data are expressed as means ± SEM (**B**–**F**). $*P < 0.05$ versus Cat E Tg^+ mice (**B** and **D**–**F**), $**P < 0.01$ versus MLE12 cells transfected with pCC10-CatE (**C**). Original magnification, ×100. CTRL, MLE12 cells without Cat E treatment control; DMSO, dimethyl sulfoxide, solvent for Mdivi-1; iCat E, inducible CC10-Cat E; Mdivi-1, small molecule Drp1 inhibitor; Mdivi-1/Cat E, MLE12 cells treated with 50 μmol/L Mdivi-1 for 30 minutes followed by 100 ng/mL human recombinant active Cat E treatment for 48 hours; pCC10-CatE, Cat E overexpressing plasmid; RFU, relative fluorescence unit.

mitochondrial fission and fusion, such as Drp1 and Mfn1/2, through pathways that depend on the ubiquitin-proteasome system (UPS).[35–38] The UPS pathway is the main proteolytic system in the cytosol of eukaryotic cells. The critical protease in this pathway is the 26S proteasome, an ATP-dependent proteolytic complex, which is formed by 20S proteasome and two 19S regulatory complexes. The 20S proteasome, catalytic core of the proteasome complex, is responsible for the breakdown of key proteins involved with apoptosis, DNA repair, endocytosis, and cell cycle control.[39] We sought to investigate if Cat E regulates Parkin and the UPS pathway. We identified increased Parkin expression in both Cat E Tg mouse lungs (Figure 8A) and MLE12 cells treated with recombinant active Cat E (Figure 8B). The activation of the UPS pathway, as measured by proteasome 20S activity, was also increased in MLE12 cells treated with recombinant active Cat E (Figure 8E). To establish the relationship between Cat E and Parkin, we silenced Cat E expression by using Cat E siRNA in MLE12 cells and found that Cat E silencing decreased Parkin expression (Figure 8C). Next, we silenced Parkin in MLE12 cells and found decreased Cat E-induced Drp1 expression (Figure 8D) and proteasome 20S activity (Figure 8E). These data indicated that Cat E regulates Parkin and subsequent UPS activity and mitochondrial fission protein Drp1.

Discussion

Emphysema is characterized by loss of lung tissue and, eventually, loss of lung function. Emphysema is a main subset of COPD, a common cause of morbidity and mortality for which specific therapies are limited. Although most commonly associated with cigarette smoking, a less appreciated fact is that emphysema occurs in most nonsmokers. Inhaled irritants, such as cigarette smoke, accelerate emphysema. COPD is predicted to become the third-leading

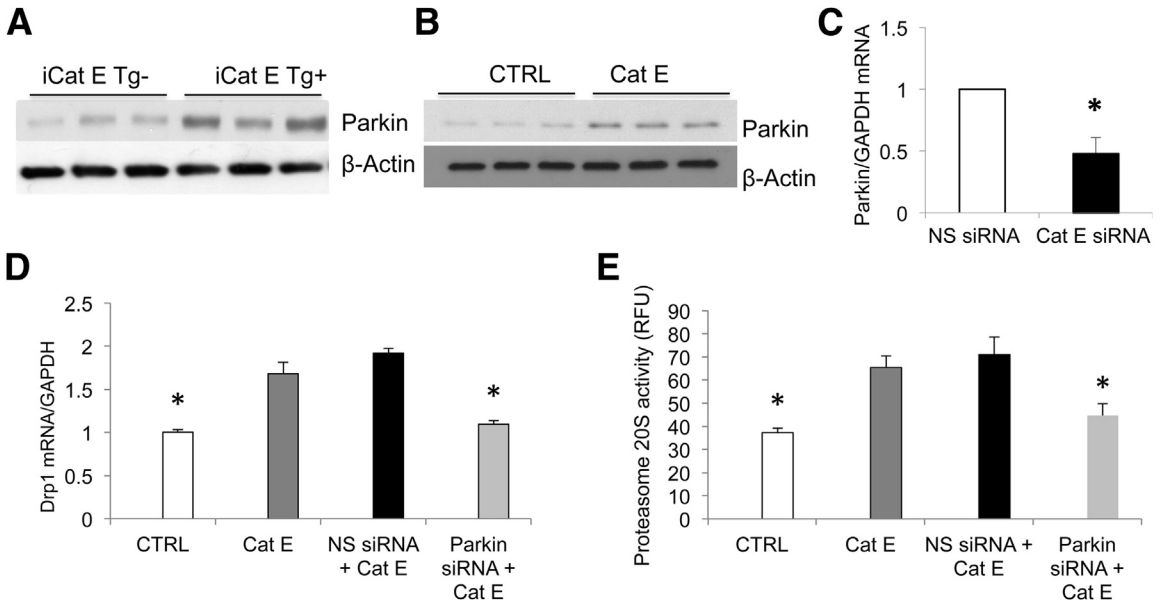

Figure 8 Parkin mediates Cat E-regulated mitochondrial fission and Drp1 induction. **A:** iCat E Tg^{+} mouse lungs (after 2 weeks of dox water) showed increased Parkin expression by Western blot analysis. **B:** MLE12 cells treated with 100 ng/mL human recombinant active Cat E for 16 hours showed increased Parkin expression by Western blot analysis. **C:** MLE12 cells transfected with 34 nmol/L NS siRNA or Cat E siRNA showed decreased Parkin expression as analyzed by real-time reverse transcription PCR. **D:** MLE12 cells transfected with 34 nmol/L NS siRNA or Parkin siRNA showed decreased Drp1 expression as analyzed by real-time reverse transcription PCR. MLE12 cells transfected with 34 nmol/L NS siRNA or Parkin siRNA showed decreased proteasome 20S activity. Data are expressed as means ± SEM. *$P < 0.05$ versus NS siRNA (**C**) or Cat E (**D** and **E**) group. CTRL, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iCat E, inducible CC10-Cat E; NS, nonspecific; RFU, relative fluorescence unit.

cause of death in 2020 because of the aging of the world population, increasing industrialization, and an expanding epidemic of smoking.[40–43] Epidemiological studies, population-based cohort studies, lung cancer screening studies, and interventional trials have also found a close relationship between COPD and lung cancer, also becoming more prevalent.[40,44] Insights into the cellular and molecular processes that govern lung tissue maintenance and destruction may yield effective therapies. To the best of our knowledge, we are the first to report that Cat E is increased in human COPD lung tissues and, when overexpressed in lung epithelial cells *in vivo*, is sufficient to cause lung emphysema. Rather than invoking Cat E's putative protease function, we found that Cat E-induced emphysema is due to increased mitochondrial fission and apoptosis. These studies highlight a novel role of Cat E and mitochondrial fission in the pathogenesis of COPD.

Cat E has been reported to be localized in different cellular compartments, such as plasma membranes, endosomal structures, the endoplasmic reticulum, and the Golgi apparatus.[9,12] In humans, Cat E can be found in dendritic cells, epithelial cells, lymphocytes, osteoclasts, spleen, and lymph nodes.[9,45,46] To date, studies have found that Cat E plays an important role in antigen processing via the major histocompatibility complex class II pathway in host defense against cancer cells, microorganisms, gastric differentiation, and development of signet-ring cell carcinoma.[8–11] Antigen processing and autoimmunity in the pathogenesis of emphysema have been reported,[47] which invokes an intriguing role for Cat E in emphysema. COPD-inducing agents, such as cigarette smoke, may stimulate Cat E expression from epithelial cells and initiate the cascade of molecular changes that lead to COPD. Our lung epithelial-targeted Tg mice and cell culture data suggest previously unidentified effects of Cat E on mitochondrial dynamics and cell death.

Unlike other cathepsins, Cat E has not been reported to exhibit proteolytic activity. In addition, endogenous substrates for Cat E have not been fully identified. Therefore, we explored non-protease mechanisms for the actions of Cat E-induced emphysema. On the basis of previous work that reported the important role of apoptosis in the pathogenesis of emphysema, we first explored if Cat E may affect apoptosis.[4,6,48] Apoptosis, or programed cell death, is mediated by caspases, a family of specific cysteine proteases. Among the members in the caspase family identified, caspase 3 is a main player in the effector phase of apoptosis induced by a variety of stimuli. Caspase 3 activation is regulated by at least two pathways: the intrinsic pathway, which involves the mitochondria (mitochondrial pathway), and the extrinsic pathway, which is stimulated by cell surface death receptors (death receptor pathway), such as tumor necrosis factor receptor 1 and Fas. The death receptor pathway of apoptosis usually leads to caspase 8 and 9 activation with subsequent activation of caspase 3. Both pathways are thought to be interlinked and converge on the mitochondria, whose outer membranes become increasingly permeable.[49] This increase in the permeability of the mitochondrial outer membrane causes proteins to leak out from the intermembranous space, activation of caspase-3 and DNases, and finally apoptosis. Given that we found increased caspase 3 activity in the

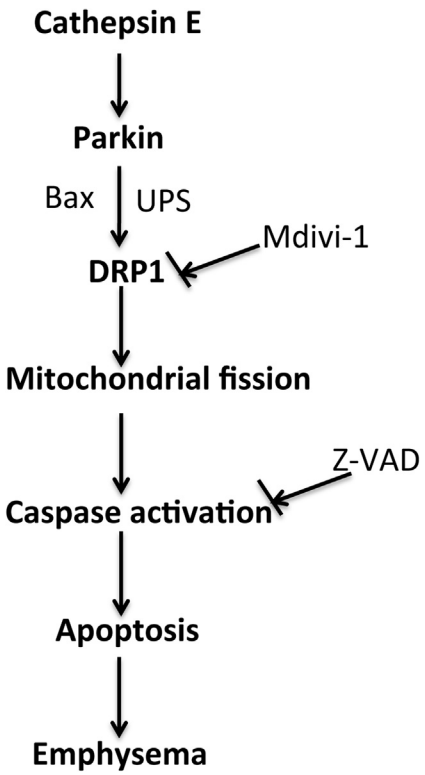

Figure 9 Summary of the effects of Cat E on emphysema. We postulate that Cat E overexpression leads to pulmonary emphysema by increasing mitochondrial fission via Parkin, UPS, and Drp1 induction, thereby disrupting the balance between mitochondrial fission and fusion, leading to increased caspase 3-mediated cell death and ultimately emphysema.

absence of increased caspase 8 and 9 activities in response to Cat E, we focused on the mitochondrial pathway. We confirmed that caspase 3 activity was part of the pathogenesis of Cat E-induced emphysema by inhibiting caspase 3 with Z-VAD, which decreased apoptosis and ameliorated emphysema in Cat E Tg mouse lungs. Next, we examined additional mitochondrial pathways that may be involved in Cat E-induced emphysema.

Mitochondria, which have long been regarded only as energy producers, are increasingly identified at the crossroads of many cellular functions.[50] Mitochondria are highly dynamic, complex organelles that continuously change their shape through two opposite processes, fission and fusion. Alterations in mitochondrial dynamics due to mutations in proteins involved in the fusion/fission machinery represent an important pathogenic mechanism of human diseases.[51] The most common proteins involved in the mitochondrial fusion process are three GTPase dynamin-like proteins: Mfn1 and Mfn2, located in the outer mitochondrial membrane, and Opa1, in the inner membrane. The most common proteins involved in the mitochondrial fission process are Drp1 and Fis1. Drp1 is a cytosolic dynamin-related GTPase which plays a central role in fission by promoting mitochondrial division through its mitochondrial outer membrane relocation and oligomerization/colocalization with other molecules, including Fis1 and Bax. We found that Cat E induces both Drp1 and Bax. Studies have found that Drp1 colocalizes with proapoptotic Bax at mitochondrial scission sites, resulting in mitochondrial fragmentation (fission) and cell apoptosis, and that these two processes occur almost simultaneously.[33,52] Inhibition of mitochondrial fission by Drp1 knock-down delays cytochrome *c* release, indicating that mitochondrial fission participates in Bax-mediated permeabilization of the outer mitochondrial membrane, caspase activation, and ultimately apoptosis.[31,53] In our models, Cat E appears to induce Drp1 and Bax, which shifts the mitochondrial balance to favor fission, thereby leading to increased mitochondrial fragmentation, cell apoptosis, and loss of lung tissue. Our observation that inhibition of Drp1 with Mdivi-1 *in vivo* completely prevented Cat E-induced apoptosis and emphysema confirms the pivotal role mitochondrial dynamics has in emphysema.

The UPS plays essential roles in regulating mitochondria dynamics.[54] Mfn1/2, Fis1, and Drp1, major regulators of mitochondrial fusion and fission, are degraded by the proteasome. MITOL, a mitochondrial E3 ubiquitin ligase, is required for Drp1-dependent mitochondrial fission because depletion or inactivation of MITOL blocks mitochondrial fragmentation.[55] Parkin, an E3 ubiquitin ligase, plays a critical role in regulating mitochondrial fission and fusion.[35] Interesting evidence links the UPS to the initiation of Parkin-dependent mitochondrial homeostasis.[37] Studies reported the involvement of Parkin in modulation of mitochondrial fusion/fission dynamics because overexpression of Parkin facilitates mitochondrial fission in mammalian primary neurons.[56] Parkin, therefore, might facilitate mitophagy by altering mitochondrial dynamics. Consistent with this notion, Parkin-mediated proteasomal degradation of mitofusins is a prerequisite for the initiation of mitophagy. Stabilization of Mfn1/2 by inhibition of the proteasome abolished the protonophores carbonyl cyanide 3-chlorophenylhydrazone-driven mitophagy.[36] Recently, Parkin-mediated mitochondrial clearance contributed to proteasome activation during denervation atrophy in slow-twitch muscles.[57] Furthermore, Parkin has also been found to regulate proapoptotic Bax by direct ubiquitination.[58] Taken together, the UPS appears to be closely linked to mitochondrial dynamics and is required for the tight regulation of Parkin-dependent mitochondrial fission/fusion. In our present study, we identified that Cat E treatment significantly increased Parkin expression and UPS activity, as measured by proteasome 20 activity. Moreover, Drp1 expression was also regulated by Cat E and Parkin, as found in siRNA studies. Taken together, we identified a novel cathepsin as an upstream inducer of Parkin, UPS activity, and Drp1-mediated apoptosis and found a link between mitochondrial fission and caspase 3-mediated apoptosis *in vivo* and *in vitro*.

It is well known that smoking is the most common identifiable risk factor for developing COPD. Here, we identified increased Cat E expression in human COPD lungs and smoking mouse lungs. Inflammation plays an important role in the pathogenesis of smoking-related COPD in both human and mouse lungs. However, emphysema can occur in conditions

with no significant inflammation in the lungs, as found in Toll-like receptor 4–deficient mouse lungs,[6] α1-antitrypsin–deficient pallid mouse lungs,[59] endothelin-2–deficient mouse lungs,[60] or klotho gene-mutated mouse lungs.[61] Similarly, Cat E-induced emphysema is not associated with overt inflammation. However, Cat E expression is increased by inflammatory stimuli, such as smoking, and may participate in the progression of COPD. Additional studies will be needed to elucidate the importance of Cat E in the pathogenesis of smoking-related COPD. Recent reports described cigarette smoke to induce mitochondrial fragmentation in airway epithelial cells and even in airway smooth muscle cells through Drp1, Opa1, or Mfn2.[62–64] However, proof of concept that Drp1 induction mediates emphysema *in vivo* has not been reported until our present studies, to the best of our knowledge. Furthermore, we identified Cat E to be an upstream mechanism of Drp1-induced emphysema and linked Cat E to Parkin and UPS activity.

Conclusions

We used *in vivo* and *in vitro* approaches to show that Cat E is a novel mediator of pulmonary emphysema. We confirmed Cat E expression is increased in both human COPD lung and smoking mouse lung tissues. We also identified previously unrecognized aspects of cathepsin biology, namely the regulation of mitochondrial fission via Parkin, UPS activation, Drp1, and downstream caspase 3 activation (Figure 9). These studies expand our understanding of the molecular mechanisms of emphysema and may provide potential therapeutic target for the prevention and treatment of emphysema.

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