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Short Communication

Cathepsin L is involved in cathepsin D processing and regulation of apoptosis in A549 human lung epithelial cells

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Abstract

Cathepsins are implicated in a multitude of physiological and pathophysiological processes. The aim of the present study was to investigate the function of cathepsin L (catL) in the proteolytic network of human lung epithelial cells and its role in the regulation of apoptosis.

We found that catL-deficient A549 cells as well as lung tissue extracts of catL$^{-/-}$ mice express increased amounts of single-chain cathepsin D (catD). Degradation experiments indicate that catL specifically degrades the single-chain isoform of catD. Furthermore, we found that catL-deficient cells showed increased sensitivity to apoptosis. Finally, we demonstrate that the inhibition of catD activity by pepstatin A decreased the number of apoptotic cells in catL-deficient A549 cells after anti-Fas treatment.

In conclusion, catL is involved in catD processing and the accumulation of catD isoforms in catL-deficient cells is associated with increased rates of spontaneous and anti-Fas-induced apoptosis.

Keywords: antisense; cysteine; mouse; protease.

Cathepsin L (catL) is a ubiquitously expressed lysosomal enzyme that belongs to the family of papain-like cysteine proteases. Among the lysosomal cathepsins of the cysteine and the aspartic protease families, catL is characterized by a very high endoproteolytic activity. Thus, the enzyme is capable of catalyzing the cleavage of a variety of functionally important substrates. Using catL$^{-/-}$ mice it was shown that catL is involved in the degradation of the invariant chain of thymic epithelial cells and, independently, of internalized self-antigens to be presented by MHC class II molecules or CD1d (Nakagawa et al., 1998; Honey et al., 2002a,b). Other targets identified to date are proenkephalin in bovine chromaffin cells (Yasothornsrikul et al., 2003), collagen XVIII (Felbor et al., 2000), and mouse thyroglobulin (Friedrichs et al., 2003). In addition, catL$^{-/-}$ mice show periodic hair loss, which is associated with hyperproliferation and defective differentiation of keratinocytes (Roth et al., 2000; Tobin et al., 2002), and also display dilated cardiomyopathy (Stypmann et al., 2002). Several studies have linked catL with apoptosis. Felbor et al. (2002) have shown that catB/L-double-'knockout' mice die early because of a massive neuronal apoptosis. In addition, Friedrichs et al. (2003) suggested that catL is important for the survival of mouse thyrocytes. The mechanisms behind this anti-apoptotic function of catL remain to be investigated.

Interestingly, increased amounts of the aspartic protease Cathepsin D (catD) have been detected in the brains of catB/L-double-'knockout' mice and in the thyroid gland of catL$^{-/-}$ mice. CatD is another potent endoprotease and several studies have linked it to apoptosis. For example, it was shown that catD is involved in the signal transduction of Fas-mediated cell death (Deiss et al., 1996). In addition, catD plays a role in p53-mediated cell death and triggers the release of cytochrome *c* from the mitochondria (Wu et al., 1998; Johansson et al., 2003).

Therefore we reasoned that the enhanced susceptibility to apoptosis in the absence of catL might be mediated by insufficient inactivation of catD.

As described previously, we have generated four subclones of the human A549 lung epithelial cell line with stable expression of catL antisense constructs that express 60–80% less catL than vector-transfected control cells (Figure 1A; Wille et al., 2002). These cell clones were used to determine whether catL is involved in catD processing and the regulation of apoptosis. First, we investigated the catD activity in the antisense-transfected cell clones using a fluorogenic substrate which is specific for catD-like proteases (Gulnik et al., 1997). In the antisense clones, we found a 2- to 10-fold increase in catD activity compared with vector-transfected control cells (Figure 1B). The concentration of defined proteins can be enhanced in cells either as a result of increased gene expression or because of the stabilization of existing proteins. To test the first hypothesis we used quantitative RT-PCR for the measurement of the catD mRNA concentration. No significant differences were found between the control cells and the antisense-transfected cells (Figure 1C). Furthermore, we analyzed the expres-

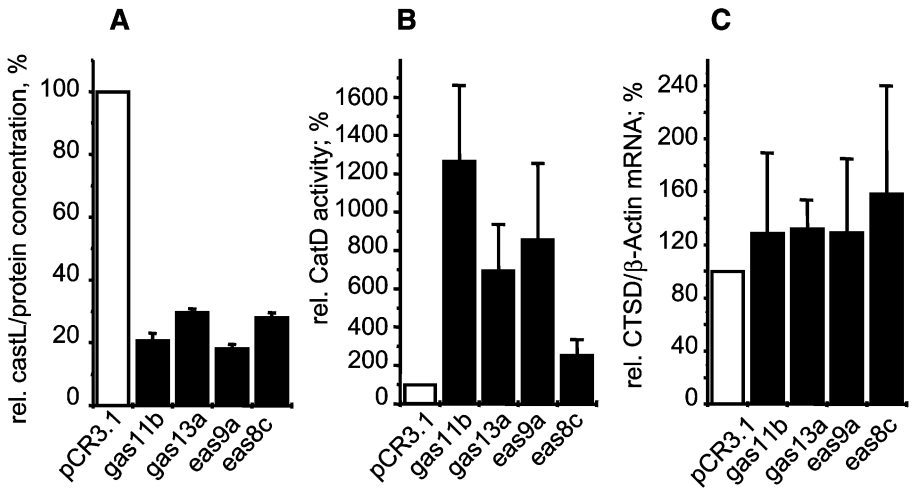

Figure 1 CatL and CatD expression in antisense-transfected A549 cells.
(A) Suppression of catL expression in cells that were stably transfected with catL-antisense constructs (gas11b, gas13, eas9a, eas8c) compared with control cells (pCR3.1). The catL concentration in the cell lysates was detemined using ELISA and normalized to the protein concentration.
(B) Increased cathepsin D (catD) activity in cells that were stably transfected with catL-antisense constructs (gas11b, gas13, eas9a, eas8c) compared with control cells (pCR3.1). The catD activity in the cell lysates was measured using the substrate Ac-Glu-Asp(EDANS)-lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Gly-Ly(DABCYL)-Glu-NH_2 in glycine-HCl buffer (pH 3.5).
(C) CatL suppression did not induce the catD mRNA concentration in antisense-transfected cells. The catD mRNA levels were measured using quantitative PCR (ABI7000, Applied Biosystems, Darmstadt, Germany). The results were normalized to β-actin expression.

sion of catD by Western blotting and found a significant increase in the amount of the enzymatically active single-chain forms of catD at 45 and 48 kDa in the antisense-transfected cells (Figure 2). We found no difference in the amount of double-chain catD, which is proteolytically active as well. Using group-specific inhibitors, Samarel et al. (1989) have shown that cysteine proteases are involved in catD processing. Bednarski et al. (1999) reported that long-term inhibition of catL is sufficient to induce the concentration of single-chain catD in hippocampal cells. We concluded from our results that single-chain catD could be specifically degraded by catL. Three sets of experiments were designed to test this hypothesis further. First, we incubated nontransfected A549 cells with the cell-permeable cysteine protease inhibitor L-*trans*-epoxysuccinyl-Leu-3-methylbutylamide-ethyl ester (E64d) and the more catL-specific inhibitor *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal (Z-Phe-Tyr-CHO). The concentration of Z-Phe-Tyr-CHO that was used in these experiments has been shown to inhibit catL activity efficiently (Woo et al., 1996). In preliminary experiments we found that incubation with 50 μM E64d inhibited 95.5±0.55% of the lysosomal/endosomal catL activity. Incubation of A549 cells with either inhibitor led to increased intracellular catD activity (Figure 3). From this result we concluded that the suppression of catL activity is indeed responsible for increased catD expression and enzymatic activity. Second, we analyzed lung tissue homogenates from $catL^{-/-}$ mice. The increased amounts of single-chain catD within the lung homogenates (Figure 4) were similar to those found in the antisense-transfected cell clones. Similar to the antisense-transfected cells, the amount of double-chain catD was not significantly changed in the samples from $catL^{-/-}$ mice. Third, we performed experiments to show that catL is able to catalyze the processing of single-chain catD. To this end, lysates of catL-deficient A549 cell clones were incubated with different concentrations of active recombinant catL. As shown in Figure 5 we found a dose-dependent decrease in the amount of single-chain catD after incubation with catL. Efficient degradation (57%) of single-chain catD at a catL concentration of 2.5 μg/ml was observed. The heavy chain of the double-chain isoform was degraded less efficiently; only 8% was degraded at the same catL concentration. These experiments demonstrated that catL is involved preferentially in the regulation of the cleavage of single-chain catD. On the other hand, the presence of significant amounts of two-chain catD in human antisense-transfected cells suggests either that catL is not the only enzyme to be involved in this process or that the remaining catL expressed in the antisense clones (ca. 20% of the control cells) is sufficient to realize a partial processing of single-chain catD. Data from mice that demonstrate the existence of two-chain catD in $catL^{-/-}$ mice indicate against a unique role of catL in the processing of single-chain catD. However, we have shown that the partial blockade of the processing of single-chain catD into its two-chain isoforms leads to delayed catD turnover and to the accumulation of catD within the antisense-transfected cells. This function of catL is not tissue-specific since increased amounts of catD were found in the brain and thyroid (Felbor et al., 2002; Friedrichs et al., 2003). However, the functional relevance of these processes remained elusive.

Several studies have described a functional role of catD in the regulation of apoptosis, hence we investigated the number of apoptotic cells in non-treated cell cultures and the sensitivity of the antisense-transfected cells to pro-apoptotic signals which were triggered by anti-Fas (CD95) antibodies. Using three different methods, which were applied in independent sets of experiments 48 h after anti-Fas treatment, we found increased amounts of

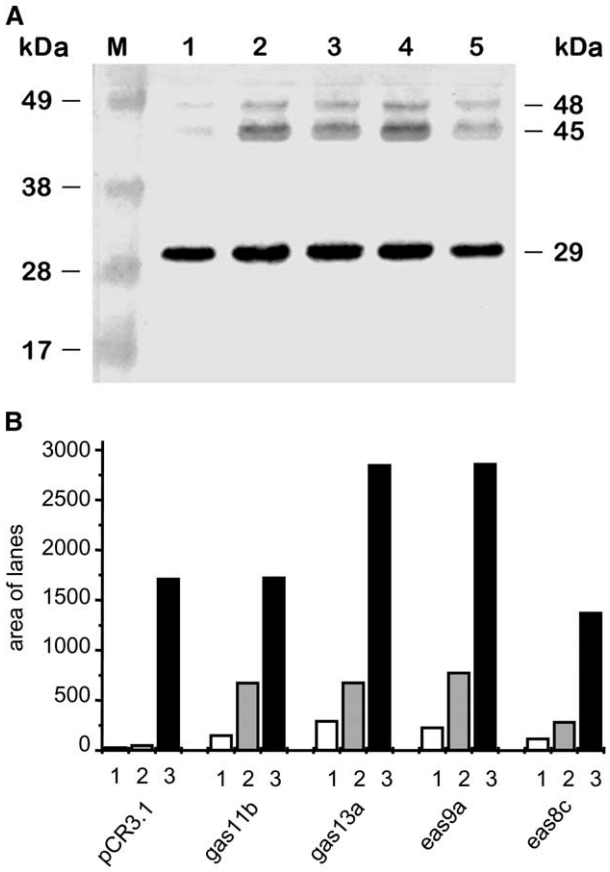

Figure 2 Increased concentration of single-chain catD in catL-antisense-transfected cells (2-gas11b, 3-gas13, 4-eas9a, 5-eas8c) compared with control cells (1-pCR3.1).
(A) Lysates (1.5 μg per lane) of the transfected cells were separated by SDS-PAGE (4–12% NuPage Gels; Invitrogen, Karlsruhe, Germany) and blotted onto a nitrocellulose membrane. CatD was labeled using a polyclonal anti-catD antibody (Wako Ltd., Osaka, Japan), polyclonal donkey anti-rabbit serum (Dianova, Hamburg, Germany), and NBT/BCIP as a substrate. The immunoreactive bands at 45/48 kDa represent single-chain catD, the band at 29 kDa represents the heavy chain of the double-chain isoform.
(B) The resulting blots were analyzed densitometrically. The areas of the bands which represented single-chain catD (48 kDa: white bars, 45 kDa: gray bars) and the heavy chain of double chain catD (29 kDa, black bars) are shown.

apoptotic cells in untreated and anti-Fas-treated, catL-deficient cells. Using the terminal desoxyribonuclease nick-end-labelling (TUNEL) technique for the detection of DNA strand breaks in untreated cell cultures, we detected a 4- to 6-fold increase in the number of apoptotic cells (Figure 6A). Following incubation of IFN-γ-sensitized cells with anti-Fas IgM in the antisense transfected cell clones, we detected 20–40% more apoptotic cells compared to the control cells (Figure 6B). Caspase 3 is a crucial executor enzyme that is activated in apoptotic cells; it cleaves a number of proteins that are important for the survival of cells and thus its activation promotes apoptosis. We found increased (60%) caspase 3 activity in anitsense-transfected cells after treatment with anti-Fas antibody (Figure 6C). Considering the relatively weak induction of caspase activity, further experiments should clarify the time course of caspase activation. In addition, we determined the expression of the 7A6 antigen; this

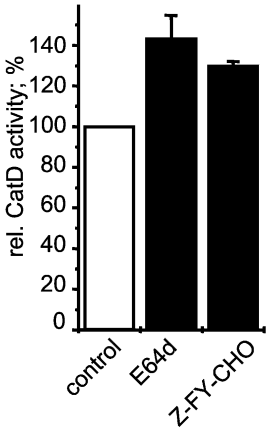

Figure 3 Suppression of catL activity using specific inhibitors induces catD activity in wild-type A549 cells.
Untransfected A549 cells were incubated for 48 h with the general inhibitor of papain-like cysteine proteases E64d (50 μM; Sigma, Deissenhofen, Germany) or the catL-specific inhibitor Z-Phe-Tyr-CHO (50 μM; Calbiochem, Bad Soden, Germany). CatD activity was measured in the cell lysates as described above.

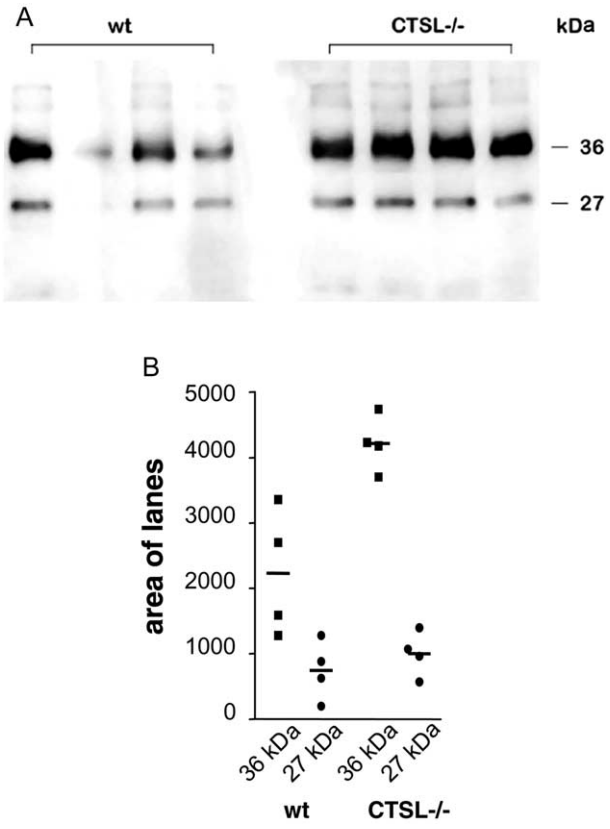

Figure 4 Increased amounts of single-chain catD in lung tissue homogenates of catL$^{-/-}$ mice.
(A) The lungs of four wild type and four catL$^{-/-}$ mice were homogenized in lysis buffer (PBS containing protease inhibitors), separated by SDS-PAGE (10%) and blotted onto nitrocellulose membranes. CatD immunoreactivity was detected as described previously (Saftig et al., 1995).
(B) The resulting blots were analyzed densitometrically. The areas of the bands which represented single-chain catD (36 kDa, squares) and the heavy chain of catD (27 kDa, circles) are shown.

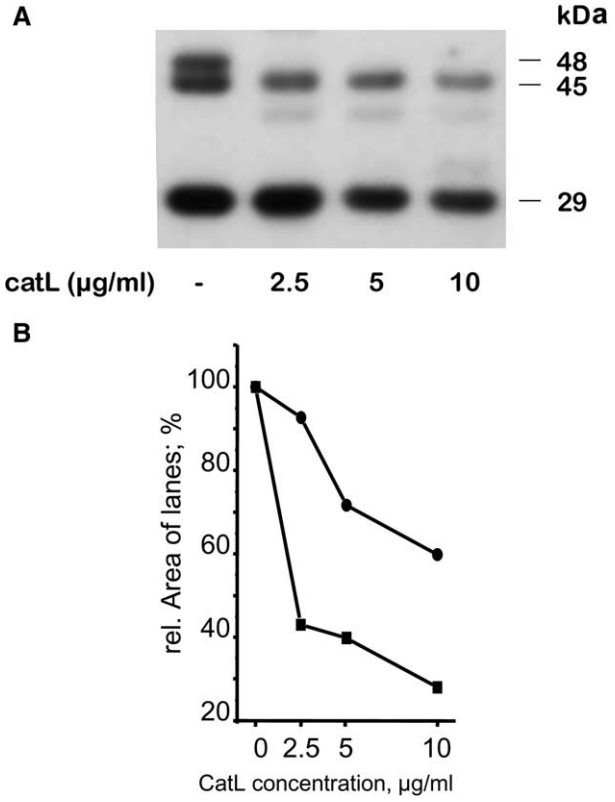

Figure 5 Recombinant catL degrades single-chain catD in a concentration-dependent manner.
(A) Lysates of antisense-transfected cells (15 µg) were incubated (1 h) in presence of different catL concentrations (lanes 2–4). In control experiments catL was omitted (lane 1). The reaction was stopped by the addition of E64 (20 µM). The reaction mixture was separated by SDS-PAGE and catD was detected as described above using ECL as a substrate (Amersham Biosciences, Freiburg, Germany).
(B) The resulting blots were analyzed densitometrically. The areas of the bands which represent single-chain catD (45/48 kDa, squares) and the heavy chain of catD (29 kDa, circles) are plotted against the catL concentrations in the reaction mixture.

antigen has previously been found on apoptotic cells (Koester et al., 1997). After anti-Fas treatment we found higher 7A6 expression on antisense-transfected cells than on control cells (Figure 6D). From these results we conclude that the suppression of catL expression leads to increased sensitivity to apoptotic signals. This could be another reason for the decreased cell growth that was previously described in these antisense-transfected A549 cells (Wille et al., 2002).

Finally, we tested whether there is an association between the catD activity and the apoptosis in the catL-antisense-transfected cells. Thus we measured apoptosis upon catD inhibition by pepstatin A, an aspartic protease inhibitor that has been shown to decrease apoptosis in several experimental systems (Thibodeau et al., 2003; Johansson et al., 2003). The antisense-transfected cells and the control cells were incubated in presence of pepstatin A (100 µM) for 24 h. These conditions blocked 66.8±14.5% of the initial catD activity. Next, apoptosis was induced by IFN-γ and anti-Fas antibody. We found no significant increase of the catD activity after IFN-γ/anti-Fas treatment. The resulting number of apoptotic cells was decreased by 65±8.7% (Figure 7). From this finding we conclude that apoptosis depends in part on the catD activity within the cells and that the increased catD activity in the antisense-transfected cells clones could mediate the enhanced sensitivity to apoptotic signals.

In conclusion, we provide evidence that catL plays an important role in the processing and therefore in the turnover of catD. Suppression of catL activity leads to an accumulation of catD. We have thus shown that active catL is necessary for the regulation of the intralysosomal homeostasis at the level of protein processing. We suggest that single-chain catD is more resistant than the two-chain form to proteolytic degradation by other proteases; this causes the accumulation of catD in a situation where the processing of the single-chain isoform by

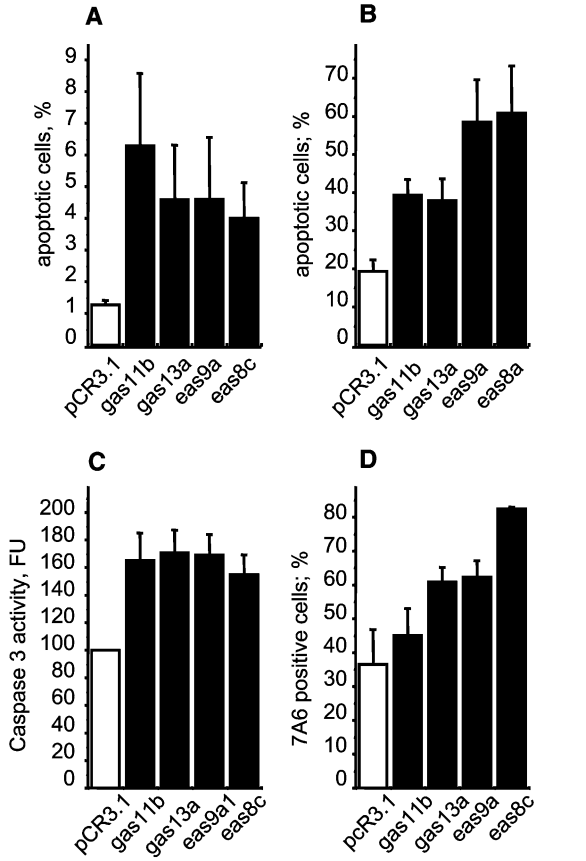

Figure 6 Enhanced apoptosis in catL-antisense-transfected A549 cells.
(A) Apoptosis was measured using TUNEL staining (ApoBrdU kit; Pharmingen, Heidelberg, Germany) in untreated A549 cells that were transfected with catL-antisense constructs (gas11b, gas13, eas9a, eas8c) or empty vector (pCR3.1). The number of apoptotic cells was measured using flow cytometry (FACS Calibur; BD, Heidelberg, Germany).
(B) Apoptosis was measured using TUNEL staining in cells that were incubated with IFN-γ (100 U, 12 h) and anti-Fas IgM (CH11, 48 h; Biozol, Eching, Germany).
(C) Caspase 3 activity in cell lysates was measured after incubation with IFN-γ and anti-Fas antibody using the substrate Z-Asp-Glu-Val-Asp -AMC with help of a fluorescence plate reader (Fluorolite; Dynatech Laboratories, Chantily, USA).
(D) The expression of the 7A6 protein during INF-γ/anti-FAS treatment was measured after incubation of the transfected cells with the antibody Apo2.7-PC5 (Beckmann/Coulter, Krefeld, Germany) using flow cytometry.

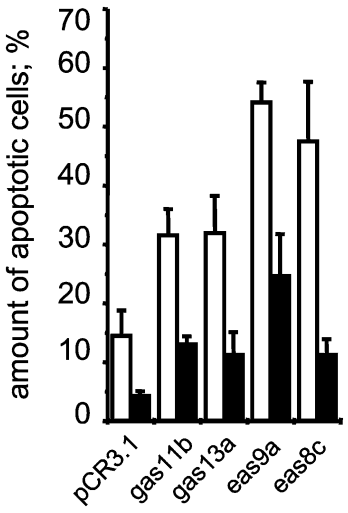

Figure 7 Apoptosis is suppressed in antisense-transfected cells after incubation with the catD inhibitor pepstatin A.
Cells were incubated with (white bars) or without (black bars) pepstatin A (100 μM, 24 h). Apoptosis was induced by IFN-γ/anti-Fas antibody treatment. The apoptotic cells were labeled using TUNEL staining.

catL is delayed. Furthermore, we have shown that the enhanced catD activity leads to increased apoptosis in catL-deficient lung epithelial cells. The molecular mechanisms that induce apoptosis after catD accumulation have not yet been investigated in detail. However, a number of studies have linked catD to apoptosis. Deiss et al. (1996) have shown that overexpression of catD induces cell death in the absence of any external stimulus. Furthermore, Brunk et al. (1999) have more recently documented that Fas-mediated signals are accompanied by lysosomal leak. The antisense-transfected cells used in our study accumulated active catD in their lysosomes and therefore were able to release more catD into the cytoplasm where the enzyme could mediate cytochrome *c* release and caspase activation (Johansson et al., 2003). We suggest that the catL-mediated catD processing is relevant *in vivo* and is not restricted to lung tissue because the reports by Felbor et al. (2002) and Friedrichs et al. (2003) have shown that decreased catL activity promotes apoptosis in the brain and the thyroid, and because we have found that the amount of catD was increased in the lung tissue of $catL^{-/-}$ mice. Since lungs of $catL^{-/-}$ mice are morphologically normal, further studies should address the functional relevance of the altered proteolytic network in lung epithelium upon pathological challenge.

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