

Progranulin Stimulates the *In Vitro* Maturation of Pro-Cathepsin D at Acidic pH

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Abstract

Single-copy loss-of-function mutations in the progranulin gene (*PGRN*) underlie the neurodegenerative disease frontotemporal lobar degeneration, while homozygous loss-of-function of *PGRN* results in the lysosomal storage disorder neuronal ceroid lipofuscinosis. Despite evidence that normal *PGRN* levels are critical for neuronal health, the function of this protein is not yet understood. Here, we show that *PGRN* stimulates the *in vitro* maturation of the lysosomal aspartyl protease cathepsin D (CTSD). CTSD is delivered to the endolysosomal system as an inactive precursor (proCTSD) and requires sequential cleavage steps *via* intermediate forms to achieve the mature state (matCTSD). In co-immunoprecipitation experiments, *PGRN* interacts predominantly with immature pro- and intermediate forms of CTSD. *PGRN* enhances *in vitro* conversion of proCTSD to matCTSD in a concentration-dependent manner. Differential scanning fluorimetry shows a destabilizing effect induced by *PGRN* on proCTSD folding ($\Delta T_m = -1.7$ °C at a 3:1 molar ratio). We propose a mechanism whereby *PGRN* binds to proCTSD, destabilizing the propeptide from the enzyme catalytic core and favoring conversion to mature forms of the enzyme. Further understanding of the role of *PGRN* in CTSD maturation will assist in the development of targeted therapies for neurodegenerative disease.

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Introduction

Progranulin (*PGRN*) is a highly conserved glycoprotein implicated in diverse biological processes [1–3]. Loss-of-function mutations in one copy of the progranulin gene (*PGRN*) cause the adult-onset neurodegenerative disease frontotemporal lobar degeneration [4,5], whereas homozygous null mutations result in the childhood-onset lysosomal storage disorder, neuronal ceroid lipofuscinosis [6]. In addition, *PGRN* is over-expressed in cancerous cells, where it can promote tumor growth and metastasis [7–9]. This suggests an important role for *PGRN* in regulating cellular growth, lysosome function, and neuronal health. Indeed, loss of *PGRN* results in dysfunctional lysosomes [10–12], and *PGRN* depletion has a beneficial effect on disease pathology in animal models of both Alzheimer's [13] and Parkinson's disease [14].

Cathepsin D (CTSD) is a lysosomal aspartyl protease with nearly ubiquitous tissue expression [15,16]. In striking parallel to *PGRN*, CTSD loss-of-function is implicated in lysosomal storage disorders [17,18] and neurodegeneration [19,20]. In addition, it is over-expressed and secreted from multiple cancerous cell types [21,22], where it stimulates invasion and metastasis [23–25]. CTSD is synthesized as an inactive pro-protein (proCTSD; ~52 kDa) and undergoes several proteolytic processing steps to a mature active protease (matCTSD; ~30 kDa) in the lysosomal compartment [26]. These include removal of a 44-amino-acid propeptide [26] and the cleavage of the single-chain protein into a ~30-kDa heavy chain and a ~15-kDa light chain, held together in the mature enzyme through non-covalent interactions [26–28].

The propeptide of CTSD is required for proper folding of the proenzyme and lysosomal targeting

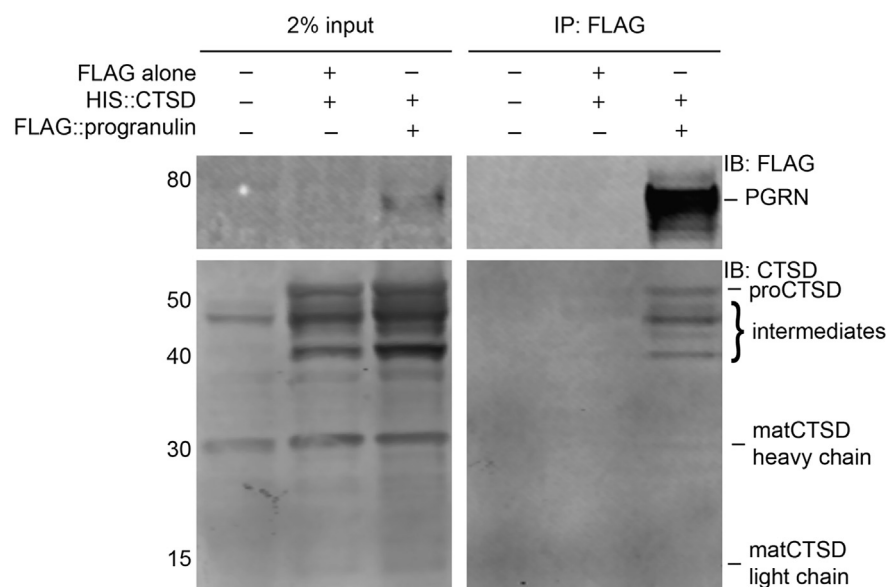


Fig. 1. PGRN binds predominantly to pro- and intermediate forms of CTSD. Pull-down of proCTSD with PGRN expressed in HEK293FT cells. Immunoprecipitation was performed against the FLAG epitope on FLAG-tagged PGRN. Immunoblotting was performed with anti-FLAG and anti-CTSD antibodies. Immunoblots are representative of three independent experiments.

and ensures that the enzyme remains inactive until activation in the appropriate environment [29]. It obstructs access to the active site cleft, thereby preventing protease activity [30,31], although there is evidence of proCTSD activity on small peptides [32]. The propeptide can be removed by cysteine proteases *in vivo* [28,33,34] and in an autocatalytic manner triggered by acidification *in vitro* [32,35,36]. An exon 2 polymorphism within the propeptide region of CTSD can disrupt normal maturation of proCTSD and has been associated with neurodegenerative disease [20,37,38]. This Ala38Val substitution results in higher proCTSD secretion, reduced maturation of the enzyme [38], and increased levels of beta-amyloid [39] and tau protein [37] in Alzheimer's disease.

Recently, several studies have reported an interaction between PGRN and CTSD [40–42] and have proposed that PGRN may stimulate CTSD protease activity [40,42]. Indeed, CTSD activity is reduced in both mouse and patient-derived cell models of PGRN deficiency [40–43]. Despite these advances, the molecular basis of the CTSD/PGRN interaction remains elusive. In this study, we demonstrate that PGRN induces a destabilizing effect on proCTSD, which promotes its maturation to the enzymatically active mature form. We propose a kinetic model whereby PGRN binds the CTSD propeptide to destabilize its interaction with the enzyme catalytic core. Our results suggest a potential mechanism for PGRN deficiency contributing to lysosomal dysfunction and neuronal cell loss.

Results and Discussion

PGRN binds predominantly to pro-forms of CTSD in a co-immunoprecipitation assay

Several recent studies have shown that PGRN can bind to CTSD, where it may function to regulate CTSD stability and protease activity [40–42]. We confirmed this interaction through the expression and co-immunoprecipitation of tagged PGRN and proCTSD in HEK293FT cells (Fig. 1). Here, we noted that primarily pro- (~52 kDa) and intermediate (~40–50 kDa) forms of CTSD bind to PGRN, rather than the final mature forms of CTSD, consisting of the heavy (~30 kDa) and light (~15 kDa) chains. This was a first indication that PGRN may bind to CTSD primarily *via* the propeptide and could thereby effectively function in regulating the maturation of proCTSD to matCTSD. Such a preferential interaction with proCTSD has also been shown for prosaposin [44], a lysosomal protein of modulatory structure like PGRN [45], which is known to interact with PGRN [46,47].

PGRN reduces the melting temperature of proCTSD through a destabilizing effect

As our immunoprecipitation results suggest a specific interaction between PGRN and proCTSD, we sought to evaluate how this interaction impacts the conformational stability of proCTSD *in vitro*.

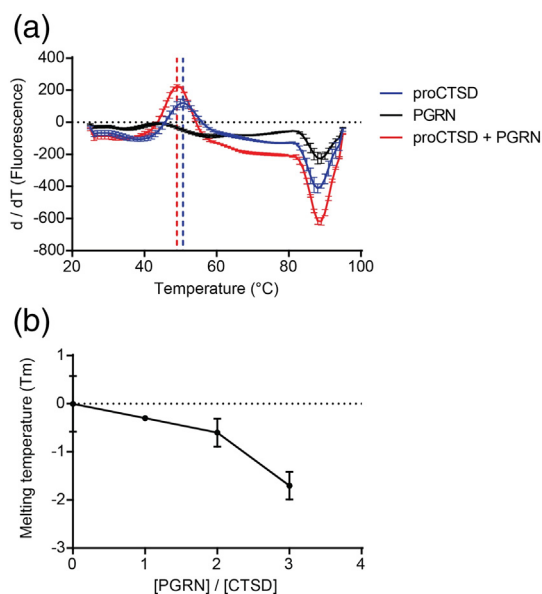


Fig. 2. PGRN reduces the melting temperature of proCTSD through a destabilizing effect. (a) DSF was used to obtain fluorescent intensity curves *versus* temperature, and the curve derivatives are plotted for recombinant proteins: 1.5 μ M HIS-tagged proCTSD alone (blue), 4.5 μ M HIS-tagged PGRN alone (black), and 1.5 μ M proCTSD with 4.5 μ M PGRN (red). DSF was performed at neutral pH 7.4. Assays were run in triplicate, and values plotted are mean \pm SEM. (b) Melting temperature, T_m , for PGRN: proCTSD complex at increasing molar ratios of PGRN.

Differential scanning fluorimetry (DSF) is a technique that can measure the melting temperature (T_m), or stability, of recombinant proteins alone or in complexes. We first confirmed the purity and correct molecular weight of commercially available recombinant PGRN and proCTSD proteins by silver stain and SDS-PAGE (Fig. S1). We next performed DSF on these proteins at neutral pH to assess their stability in the absence of auto-activation of proCTSD to matCTSD. When subjected to DSF, PGRN alone did not show an unfolding transition on increasing temperature (Figs. 2a and S2). This suggests that recombinant PGRN is thermally stable, as expected from its highly disulfide-bonded structure [48,49]. ProCTSD alone showed an unfolding transition at a T_m of 50.7 $^{\circ}$ C (Figs. 2a and S2). The addition of PGRN to proCTSD at a 3:1 molar ratio caused a significant destabilizing effect on the T_m of proCTSD ($\Delta T_m = -1.7$ $^{\circ}$ C) (Fig. 2a). Lower molar ratios of PGRN to proCTSD (2:1 and 1:1) resulted in a concentration-dependent temperature shift of -0.6 $^{\circ}$ C and -0.3 $^{\circ}$ C, respectively (Figs. 2b and S2).

We noted that the unfolding curve for the proCTSD:PGRN complex presented with a larger change in fluorescence than for proCTSD alone ($\Delta F_{\text{proCTSD:PGRN}} > 1500$ a.u.; $\Delta F_{\text{proCTSD}} < 1000$ a.u.) (Fig. S2A), suggesting an increase in exposure of

proCTSD hydrophobic residues and cooperativity in unfolding in the presence of PGRN. Interestingly, a similar mechanism of action has been proposed for sulfated polysaccharides on both aspartyl [31] and cysteine proteases [50–52], whereby destabilization of the propeptide favors its cleavage. These negatively charged compounds are hypothesized to interact with Arg3 and Arg11 residues of the CTSD propeptide, reducing their electrostatic interaction with residues Asp181 and Asp12 of the enzyme catalytic core [31].

PGRN increases the conversion rate of proCTSD to matCTSD

Given that PGRN binds to and destabilizes proCTSD, we next evaluated a potential role for PGRN in proCTSD maturation at an acidic pH of 3.4. In the presence of PGRN, we noted an increase in the formation of matCTSD (Fig. 3a–d). We observed the same result with a differentially tagged recombinant PGRN purchased from an alternate source (Fig. S3). To test for a concentration-dependent effect of PGRN on proCTSD conversion to matCTSD, we estimated the kinetics of maturation from the immunoblot signals. First, we assessed whether there was a concentration-dependent conversion of proCTSD to matCTSD in the absence of PGRN. Indeed, we observed a concentration dependence in the maturation of proCTSD alone (Fig. S4). Calculation of the initial velocities (V_0) of these reactions with increasing proCTSD concentration demonstrated that V_0 increases non-linearly with proCTSD concentration, consistent with a quadratic relationship (Appendix 1), as predicted from an intermolecular activation mechanism (Fig. S4). We next calculated V_0 for proCTSD maturation in the presence of increasing concentrations of PGRN. We found that V_0 increased with increasing PGRN concentration (Fig. 3e–f), confirming a concentration-dependent increase in proCTSD maturation in the presence of PGRN. Both PGRN and proCTSD undergo direct trafficking to the lysosome *via* the mannose-6-phosphate receptor pathway [47,53,54]. It is possible that at least part of this trafficking may occur in complex together. On reaching the lysosome, this may allow PGRN to regulate the maturation and/or activity of the protease. Such a role for PGRN in proCTSD maturation may help to explain the impaired CTSD activity observed in models of PGRN deficiency [40–42]. Indeed, CTSD activity (V_{max}) was increased by approximately 50% in the presence of PGRN at a 3:1 molar ratio (Fig. S5).

A kinetic model for the role of PGRN in proCTSD maturation

ProCTSD undergoes *in vitro* maturation to its heavy (~30 kDa) and light (~15 kDa) chains at acidic pH, a process that involves the cleavage of the 44-residue

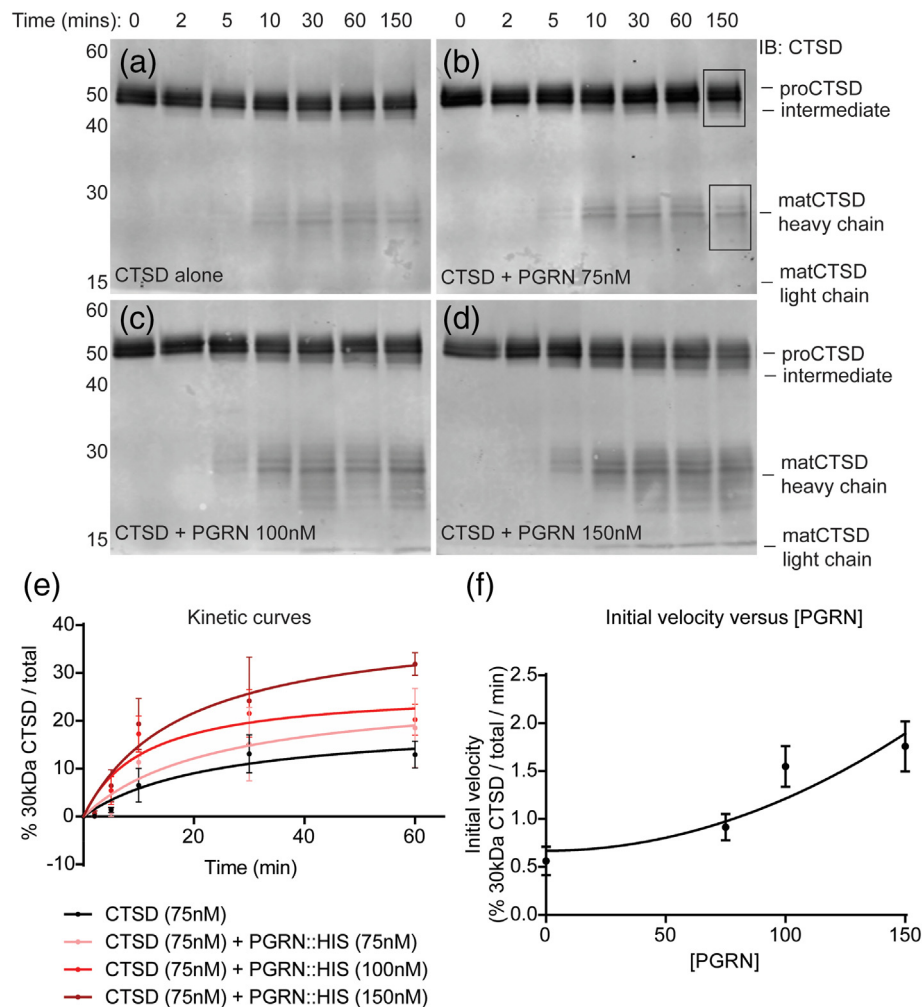


Fig. 3. PGRN increases the conversion rate of proCTSD to matCTSD. (a–d) *In vitro* maturation time-course assays for recombinant HIS-tagged proCTSD at pH 3.4 in the (A) absence ($n = 4$) and presence of (b) 75 nM ($n = 3$), (c) 100 nM ($n = 3$), and (d) 150 nM ($n = 3$) HIS-tagged PGRN. Membranes were immunoblotted with anti-CTSD antibody. Boxed regions in panel B indicate the signal measured for quantification of % 30 kDa CTSD/total. (e) Michaelis–Menten kinetic curves for the conversion of proCTSD to matCTSD. (f) Correlation between initial velocity of reaction (V_0) and PGRN concentration.

propeptide [32]. Initially, the first molecules of proCTSD are processed to matCTSD through a slow intramolecular cleavage mechanism [32]. The newly formed matCTSD then accelerates the maturation process further by cleaving the propeptide from proCTSD through an intermolecular activation mechanism [32]. We developed a simple kinetic model for the role of PGRN in converting proCTSD to matCTSD (Appendix 1) and hypothesize that destabilizing the interaction of the propeptide with the enzyme catalytic core facilitates its cleavage by matCTSD molecules (Fig. 4).

Our kinetic model suggests that the concentration of matCTSD in cells would depend on the concentration of PGRN (Appendix 1). As such, loss-of-function mutations in PGRN would be expected to impair the maturation of proCTSD to matCTSD

and thus reduce protease activity. Such a reduction in CTSD enzymatic activity has been observed in several recent studies [40–43]. Despite these observed impairments in CTSD enzymatic activity, studies also report an increase in pro-CTSD and/or matCTSD expression levels on loss of PGRN [40,42,55–57]. It is possible that these changes in CTSD expression level represent a compensatory transcriptional response to promote lysosome function [58,59]. Such cellular compensatory responses highlight the importance of utilizing *in vitro* assays to isolate the direct molecular effect of PGRN on CTSD. Furthermore, CTSD expression is nearly ubiquitous, but there are certain cell types where PGRN expression is low (e.g., neurons, muscle, adipose, soft tissue). As CTSD can undergo a slow auto-activation mechanism by itself at acidic pH, it is

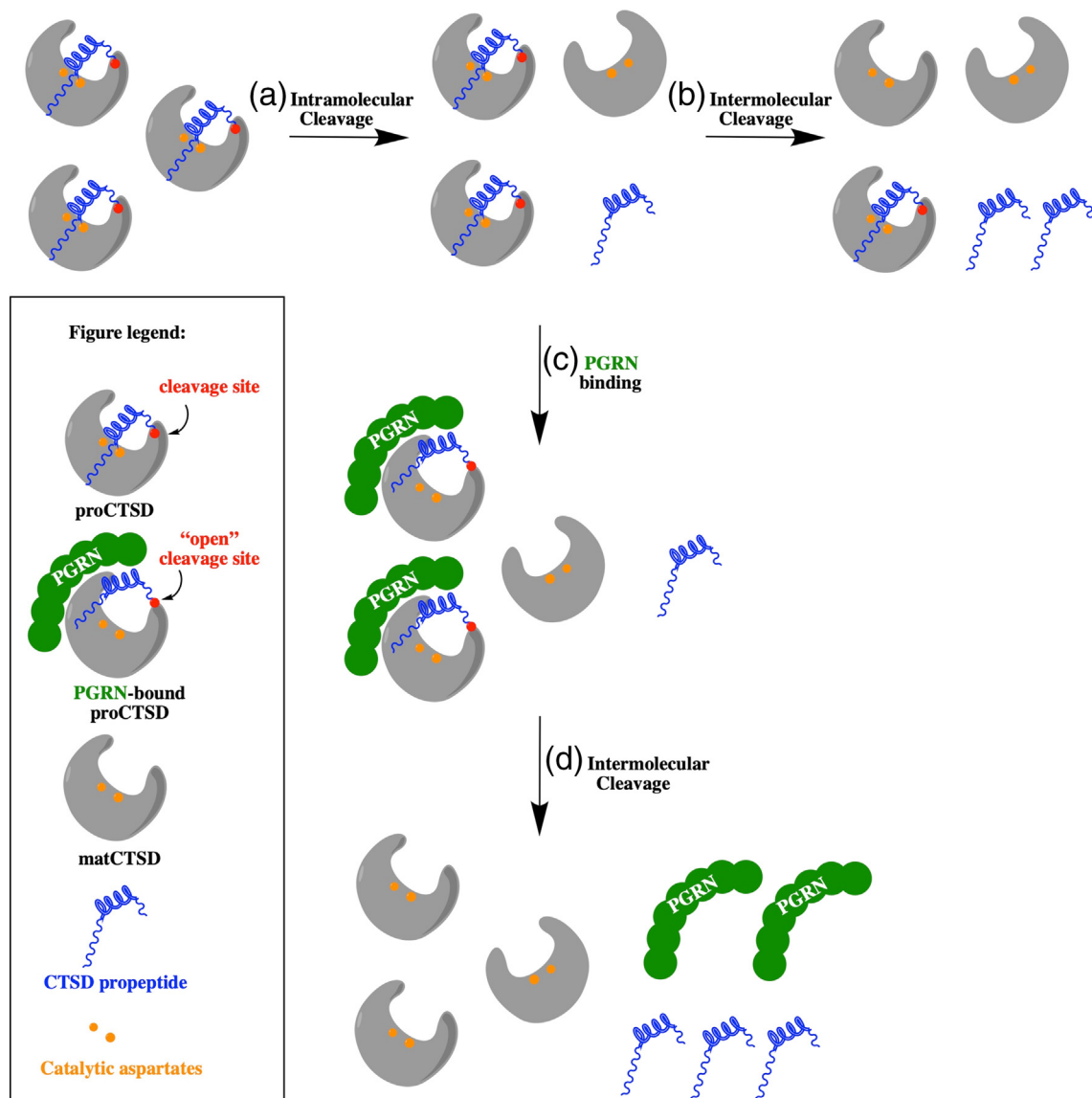


Fig. 4. A model for the role of PGRN in the conversion of proCTSD to matCTSD. (a) ProCTSD undergoes an autocatalytic activation mechanism for the formation of initial matCTSD molecules at a low rate. (b) Once formed, matCTSD can convert proCTSD to matCTSD through an intermolecular cleavage of the propeptide. (c) PGRN binds around the propeptide region of proCTSD to destabilize its interaction with the enzyme catalytic core, (d) facilitating propeptide cleavage by matCTSD. Catalytic aspartyl residues are represented as orange dots, the propeptide in blue, and the propeptide cleavage site in red.

likely that the presence of PGRN for CTSD maturation is not an absolute requirement in all cell types under all conditions. PGRN expression may perhaps be a regulatory mechanism to “tune up” CTSD activity. Furthermore, PGRN is a secreted glycoprotein, and therefore, cell autonomous expression is not required in order for PGRN to affect protease function. We also cannot exclude other mechanisms regulating CTSD maturation, such as other proteases. Despite this, the data presented here suggest that the impairment of CTSD activity observed in cell

models of PGRN deficiency may result from a role for PGRN in proCTSD maturation.

Conclusion

In this work, we investigated the nature of the interaction between PGRN and proCTSD and discovered a function for PGRN in promoting the *in vitro* maturation of this aspartyl protease. DSF studies highlight a potential mechanism for this,

whereby PGRN may bind the propeptide region of proCTSD, destabilizing its interaction with the CTSD catalytic core. This may increase accessibility for intermolecular cleavage of the inhibitory propeptide, promoting formation of the active matCTSD. Further investigation will be required to determine which domains of the PGRN molecule mediate the interaction with, and stimulate the maturation of, proCTSD. A potential role for PGRN in lysosomal aspartyl protease maturation will aid in understanding of the mechanism underlying neurodegenerative diseases that are linked to PGRN loss-of-function.

Methods

HEK293FT transfections and co-immunoprecipitation assays

The cDNAs for human PGRN (a generous gift from Professor Robert Farese) and proCTSD (GenScript, No. OHu26913) were sub-cloned into the pSecTag2B vector (ThermoFisher, No. V90020). PGRN was expressed with an N-terminal FLAG tag, and proCTSD was expressed with an N-terminal His tag. HEK293FT cells (3×10^6) were seeded onto 10-cm plates and grown to 60% confluency. Total DNA (5 μ g) was transfected [Opti-MEM (ThermoFisher, No. 31985070), X-tremeGENE HP DNA Transfection Reagent (Sigma Aldrich, No. 6366236001)]. Cells were collected 48 h later and re-suspended in lysis buffer with protease inhibitor [50 mM Tris (pH 7.4; Teknova, No. T5074), 150 mM NaCl (Fisher, No. S271-3), 1% Nonidet P-40 (ThermoFisher, No. 85124), complete protease inhibitor (Roche, No. 04693124001)]. Cells were lysed for 1 h on ice and centrifuged at 20,000*g* for 20 min, and the supernatant was collected. The protein concentration of samples was measured by BCA (ThermoFisher, No. 23225). Immunoprecipitation was carried out against the FLAG tag on PGRN (Sigma Aldrich, No. M8823).

ProCTSD thermal denaturation by DSF

DSF measurements ($\lambda_{\text{ex}} = 490$ nm, $\lambda_{\text{em}} 610$ nm) were performed in 96-well plates (USA Scientific, No. 1402-9590) on a Biorad CFX96 instrument. Thermal denaturation assays (70 μ l) for 1.5 μ M proCTSD (R&D Systems Inc., No. 1014-AS) were measured in the presence and absence of PGRN (R&D Systems Inc., No. 2420-PG-050) in 50 mM Tris-HCl (Teknova, No. T5074) and 150 mM NaCl (Fisher, No. S271-3) at pH 7.4. SyproOrange (Sigma Aldrich, No. S5692) was added to all wells. Thermal denaturation curves were recorded over a temperature range from 25 to 95 °C with increments of 1 °C min⁻¹. All measurements were carried out in triplicate.

Time-dependent immunoblotting for proCTSD maturation

Assays were run in 20 μ l of 100 mM sodium citrate buffer (pH 3.4). Seventy-five nanomolar of recombinant CTSD (R&D Systems Inc., No. 1014-AS) was incubated in the absence or presence of 75, 100, or 150 nM recombinant PGRN (R&D Systems Inc., No. 2420-PG-050). Reactions were incubated at 37 °C and then immediately terminated through the addition of 4 \times LDS (ThermoFisher No., NP0007) and 10% sample reducing agent (ThermoFisher, No. NP0009). Samples were boiled at 70 °C for 10 min, analyzed by SDS PAGE on 10% gradient Bis-Tris gels (ThermoFisher, No. NP0302BOX), and transferred to PVDF membrane (Bio-Rad, No. 1620177). Membranes were immunoblotted with an anti-CTSD primary antibody (R&D Systems Inc., No. AF1014, 1:250 dilution) and a donkey anti-goat secondary antibody (LI-COR IRDye 800CW, No. 926-32,214, 1:10,000 dilution). Membranes were imaged on the LI-COR Odyssey CLx System and quantified in Licor Image Studio Lite version 5.2 software. Background-corrected signal for 30-kDa CTSD bands were divided by total signal (30 kDa + 50 kDa bands) to obtain a fraction of conversion. This was plotted against time, and points were fitted to Michaelis-Menten equation. Initial velocities were calculated using the first four time points of incubation (0, 2, 5, and 10 min). Linear regression analysis was performed in GraphPad Prism Version 6. Initial velocities were plotted against proCTSD or PGRN concentration. Silver staining of commercial recombinant protein was carried out using the SilverQuest Silver Staining Kit (Thermo Fisher, No. LC6070). The anti-paragranulin antibody was made in-house (GRN P.1, epitope TRCPDGQFCPVACCLDPGGA SYSCCRPLLD, 1:500 dilution) and used with a goat anti-mouse secondary antibody (LI-COR IRDye 800CW, No. 926-32,210, 1:10,000 dilution).

In vitro CTSD activity assays

Fluorescent protease activity assays were performed in triplicate in black, flat-bottom 384-well plates (Greiner, No. 781091). Assays were run for 2 h in 15 μ l of sodium acetate buffer (pH 3.5). CTSD (20 nM; R&D Systems Inc., No. 1014-AS) was used for all assays with 5 μ M of a fluorescent substrate that was identified previously [Lys(7-methoxycoumarin-4-acetic acid) Arg-Gly-Leu-Tyr-Phe-Ile-Thr-His-Lys(dinitrophenol)] [60]. Assays were run in the absence or presence of 65 nM PGRN (R&D Systems Inc., No. 2420-PG-050). Fluorescent substrate cleavage was monitored every 30 s with a Biotek Synergy HT plate reader using excitation and emission wavelengths of 328 and 393 nm, respectively. Linear regression analysis was performed in GraphPad Prism Version 6 on 20 consecutive time

points to calculate the maximal CTSD enzymatic activity in the presence of unlimited substrate (maximal velocity, V_{\max}). Data were normalized to the CTSD alone condition.

Acknowledgments

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Appendix 1

We developed a simple kinetic model for the role of PGRN in converting proCTSD to matCTSD. We propose a multi-step mechanism involving the destabilization of the propeptide, where openCTSD = proCTSD with destabilized propeptide:



Therefore, matCTSD can be generated through:

- 1) $\text{openCTSD} + \text{openCTSD} \rightarrow \text{openCTSD} + \text{matCTSD}$
- 2) $\text{openCTSD} + \text{matCTSD} \rightarrow \text{matCTSD} + \text{matCTSD}$

Here, we assume the destabilized propeptide may not interfere with CTSD activity, and we consider that 1) and 2) may have the kinetic rate constants $2k_1$ and k_1 , respectively. We also assume that proOPEN is catalytically active, with similar activity to matCTSD, that is, removing the auto-inhibition of the propeptide can occur reversibly by conformational changes, as well as irreversibly by cleavage.

For:

$$\frac{\delta[\text{matCTSD}]}{\delta t} = 2k_1[\text{openCTSD}]^2 \quad (1)$$

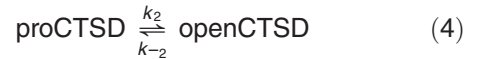
For:

$$\frac{\delta[\text{matCTSD}]}{\delta t} = k_1[\text{openCTSD}][\text{matCTSD}] \quad (2)$$

Summing (1) and (2) to calculate total matCTSD produced:

$$\frac{\delta[\text{matCTSD}]}{\delta t} = k_1[\text{openCTSD}] \times (2[\text{openCTSD}] + [\text{matCTSD}]) \quad (3)$$

In addition, proCTSD will convert to openCTSD with a kinetic rate constant of k_2 :



where

$$\frac{\delta[\text{openCTSD}]}{\delta t} = k_2[\text{proCTSD}] - k_{-2}[\text{openCTSD}] \quad (5)$$

Considering equilibrium:

$$k_2[\text{proCTSD}] - k_{-2}[\text{openCTSD}] = 0$$

$$[\text{openCTSD}] = \frac{k_2}{k_{-2}} [\text{proCTSD}]$$

Substituting for Eq. (5) into Eq. (3):

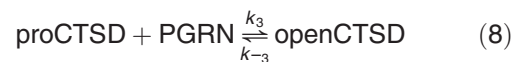
$$\frac{\delta[\text{matCTSD}]}{\delta t} = 2k_1 \cdot \left[\frac{k_2}{k_{-2}} \right]^2 [\text{proCTSD}]^2 + k_1[\text{matCTSD}] \cdot \left[\frac{k_2}{k_{-2}} \right] [\text{proCTSD}] \quad (6)$$

At the initiation of the reaction, $[\text{matCTSD}] \rightarrow 0$, therefore:

$$\frac{\delta[\text{matCTSD}]}{\delta t} \approx 2k_1 \cdot \left[\frac{k_2}{k_{-2}} \right]^2 [\text{proCTSD}]^2 \quad (7)$$

Therefore, the rate of production of matCTSD is predicted to depend quadratically on the concentration of proCTSD. The observed kinetics (Fig. S4) appears to be even "steeper" than quadratic, but it is clearly not linear.

If PGRN binds the propeptide, in the presence of PGRN;



where

$$\frac{k_3}{k_{-3}} = \frac{[\text{openCTSD}]}{[\text{proCTSD}][\text{PGRN}]} \quad (9)$$

Combining Eqs. (9) and (5):

$$\frac{k_2}{k_{-2}} = \frac{k_3}{k_{-3}} [\text{PGRN}] \quad (10)$$

Combining Eqs. (10) and (7):

$$\frac{\delta[\text{matCTSD}]}{\delta t} \approx 2k_1 \cdot \left[\frac{k_3}{k_{-3}} \right]^2 [\text{PGRN}]^2 [\text{proCTSD}]^2 \quad (11)$$

Therefore, the production of matCTSD is also predicted to depend quadratically on the concentration of PGRN, which is consistent with Fig. 3f.

Appendix 2. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2019.01.027>.

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Abbreviations used:

PGRN, progranulin; CTSD, cathepsin D; DSF, differential scanning fluorimetry.

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